

534 Rec'd PCT/PTC 18 JUL 2000

Form PTO-1390 (REV 11-98)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER 160180.90121
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371			US. APPLICATION NO. (If known, see 37 CFR 1.5) 09/600535
INTERNATIONAL APPLICATION NO. PCT/US99/01087	INTERNATIONAL FILING DATE 19/01/1999	PRIORITY DATE CLAIMED 20/01/1998	
TITLE OF INVENTION METHODS AND COMPOSITIONS TO ALTER TISSUE SUSCEPTIBILITY TO IMMUNE INJURY, TO PROGRAMMED CELL DEATH, AND TO CLEARANCE BY THE RETICULOENDOTHELIAL SYSTEM.			
APPLICANT(S) FOR DO/EO/US Peter J. Sims, Therese Wiedmer, Ji Zhao			
<p>Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:</p> <ol style="list-style-type: none"><input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.<input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.<input type="checkbox"/> This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).<input checked="" type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.<input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2))<ol style="list-style-type: none"><input type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau).<input type="checkbox"/> has been transmitted by the International Bureau.<input checked="" type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US)<input type="checkbox"/> A translation of the International Application into English (35 U.S.C. 371(c)(2)).<input type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))<ol style="list-style-type: none"><input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau).<input type="checkbox"/> have been transmitted by the International Bureau.<input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired.<input checked="" type="checkbox"/> have not been made and will not be made.<input type="checkbox"/> A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).<input type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).<input type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)). <p>Items 11. to 16. below concern document(s) or information included:</p> <ol style="list-style-type: none"><input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98.<input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.<input checked="" type="checkbox"/> A FIRST preliminary amendment. <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment.<input type="checkbox"/> A substitute specification.<input type="checkbox"/> A change of power of attorney and/or address letter.<input checked="" type="checkbox"/> Other items or information: return receipt postcard.			

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09/600535

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

U.S. National Phase Application Corresponding To:

PCT Application: PCT/US99/01087
International
Filing Date: 12 JAN 1999
Applicant: Peter J. Sims et al.
Title: METHODS AND COMPOSITIONS TO ALTER TISSUE
SUSCEPTIBILITY TO IMMUNE INJURY, TO PROGRAMMED
CELL DEATH, AND TO CLEARANCE BY THE
RETICULOENDOTHELIAL SYSTEM
Atty. Docket No.: 160180.90121

BOX PCT
Commissioner for Patents
Washington, D.C. 20231

PRELIMINARY AMENDMENT

Dear Sir:

Prior to examination of the above-identified U.S. national phase application, Applicants respectfully request that the following amendment be entered.

In the Claims

Please amend the claims as follows:

21. (Amended) The methods of claim 19 [claims 19 or 20] wherein the level of PL scramblase RNA is measured by *in situ* hybridization or Northern blotting with PL scramblase cDNA or by the polymerase chain reaction using oligonucleotide primers based on sequence of human PL scramblase cDNA.

22. (Amended) The method of claim 19 [claims 19 or 20] wherein the level of PL scramblase protein is measured by immunofluorescence, flow cytometry, Western blotting, radioimmunoassay, or by ELISA assay using antibody that specifically binds PL scramblase protein.

Please add the following new claims 29-30:

--29. The methods of claim 20 wherein the level of PL scramblase RNA is measured by *in situ* hybridization or Northern blotting with PL scramblase cDNA or by the polymerase chain reaction using oligonucleotide primers based on sequence of human PL scramblase cDNA

30. The method of claim 20 wherein the level of PL scramblase protein is measured by immunofluorescence, flow cytometry, Western blotting, radioimmunoassay, or by ELISA assay using antibody that specifically binds PL scramblase protein.--

Remarks

The above amendments are being made and two new claims are added to eliminate multiple dependencies in the claims. No new matter is added.

No fee is believed necessary to enter this amendment. However if a fee is necessary, please charge Deposit Account 17-0055.

Respectfully submitted,

Dated: July 18, 2000

By: Jean C Baker

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**VERIFIED STATEMENT CLAIMING SMALL ENTITY STATUS
(37 CFR 1.9(f) and 1.27(c))--NONPROFIT ORGANIZATION**

Docket Number (Optional)
160180.90121

Applicant or Patentee: Peter J. Sims et al.

Serial or Patent No.: 09/600,535

Filed or Issued: July 18, 2000

Title: METHODS AND COMPOSITIONS TO ALTER TISSUE SUSCEPTIBILITY TO IMMUNE INJURY, TO PROGRAMMED CELL DEATH, AND TO CLEARANCE BY THE RETICULOENDOTHELIAL SYSTEM

I hereby declare that I am an official of empowered to act on behalf of the nonprofit organization identified below:

NAME OF NONPROFIT ORGANIZATION: The Blood Center of Southeastern Wisconsin

ADDRESS OF NONPROFIT ORGANIZATION: 8727 Watertown Plank Road
Milwaukee, WI 53201-2178

TYPE OF ORGANIZATION

☐ University or Other Institution of Higher Education

☒ Tax Exempt Under Internal Revenue Code (26 USC 501(a) and 501(c)(3))

☐ Nonprofit Scientific or Educational Under Statute of State of the United States of America

Name of State: _____

Citation of Statute _____

☐ Would Qualify as Tax Exempt Under Internal Revenue Code (26 USC 501(a) and 501(c)(3)) if Located in the United States of America

☐ Would Qualify as Nonprofit Scientific or Educational Under Statute of a State or the United States of America if Located in the United States of America

Name of State: _____

Citation of Statute _____

I hereby declare that the above identified nonprofit organization qualifies as a nonprofit organization as defined in 37 CFR 1.9(e) for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code, with regard to the invention described in:

☐ the specification filed herewith with the title as listed above.

☒ the application identified above.

☐ the patent identified above.

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization identified above with regard to the above-identified invention. If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights in the invention must file separate verified statements averring to their status as small entities and that no rights to the invention are held by any person, other than the inventor, who could not qualify as an independent inventor under 37 CFR 1.9(c) if that person made the invention, or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

Each such person, concern or organization having rights in the invention is listed below:

☒ No such person, concern, or organization exists.

☐ Each such person, concern or organization is listed below.

FULL NAME _____

ADDRESS _____

Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING Richard H. Aster

TITLE IN ORGANIZATION OF PERSON SIGNING Intellectual Property Coordinator

ADDRESS OF PERSON SIGNING 8727 Watertown Plank Road, Milwaukee, Wisconsin 53201-2178

SIGNATURE

Richard H. Aster

DATE 8-19-00

METHODS AND COMPOSITIONS TO ALTER TISSUE SUSCEPTIBILITY
TO IMMUNE INJURY, TO PROGRAMMED CELL DEATH, AND TO
CLEARANCE BY THE RETICULOENDOTHELIAL SYSTEM

CROSS-REFERENCE TO RELATED APPLICATION

This application claims priority from U.S. Serial
No. 60/071,950, filed January 20, 1998.

5 STATEMENT REGARDING FEDERALLY SPONSORED
RESEARCH OR DEVELOPMENT

United States Government may have commercial rights
under Grant R01 HL36946 from Heart, Lung, & Blood
Institute, National Institutes of Health.

BACKGROUND OF THE INVENTION

10 As described in U.S. Serial No. 08/790,186, cell
surface PS has a role in coagulation, programmed cell
death and clearance by the reticuloendothelial system.
U.S. Serial No. 08/790,186 also describes regulation of
the transmembrane distribution of PS, the role of calcium
15 in the collapse of phospholipid asymmetry, and the role
PL translocation in Scott Syndrome.

Programmed Cell Death (Apoptosis)

Programmed cell death or apoptosis is an important
process that is now recognized to be central to the
20 selective elimination of mammalian cells during
embryogenesis, tissue remodeling, the clonal selection of
immune cells, metamorphosis and hormone-dependent
atrophy, as well as cell death induced by chemicals or
irradiation (reviewed by G.M. Cohen, Biochem. J. 326:1-
25 16, 1997; D.L. Vaux and A Strasser, Proc. Natl. Acad.

Sci. USA 93:2239-2244; D. J. McConkey and S. Orrenius, Biochem Biophys. Res. Commun. 239:357-366, 1997, and references therein). Inappropriate apoptosis (either unregulated excessive or inappropriately low rate) is now

5 implicated in many human diseases, including Alzheimer's and Huntington's disease, autoimmune disorders, ischemic damage, and several forms of cancer (M.E. Peter, et al., Proc. Natl. Acad. Sci. USA 94:12736-12737, 1997).

Although the specific pathways leading to cell death may

10 vary for different cell types and cell stimuli, it is convenient to divide the process into distinct phases. The earliest phase is the stimulus that initiates the apoptotic response. This may be an external signal delivered through surface receptors such as CD95 (Fas) or

15 tumor necrosis factor receptor, or may originate inside the cell in response to a drug, irradiation or toxin. In the next phase, the signal is detected and transduced to the cell death effector machinery. The third phase of the apoptotic process includes activation of proteases

20 (including caspases), as well as their positive and negative regulators (including Bcl-2, Bcl-x_L, and their inhibitors Bax, Bad, and Bak) (J.C. Reed, Nature 387:773-776, 1997; T. Patel, et al., FASEB J. 10:587-597, 1996; G. M. Cohen, supra, 1997). Activation of the "execution

25 phase" results in morphological changes including cell shrinkage, cell surface exposure of aminophospholipids resulting in the recognition and phagocytosis of apoptotic cells, and plasma membrane blebbing. In the

last phase, chromatin condensation and degradation of DNA is observed. Internucleosomal degradation of DNA into multiples of 180-200 base pair fragments, recognized as "DNA laddering", has long been considered a biochemical
5 hallmark of apoptosis, although more recent work has shown that the formation of larger DNA fragments, most notably of 50 and 300 kilobases, precedes oligonucleosomal DNA fragmentation in all systems investigated (A.H. Wyllie, Nature 284:555-556, 1980; D.G.
10 Brown, et al., J. Biol. Chem. 268:3037-3039, 1993).

Phosphatidylserine Exposure in Apoptosis

In recent years it has become clear that externalization of plasma membrane phosphatidylserine (PS) is universally observed in cells undergoing
15 apoptosis, although the signal transduction pathways leading to surface exposure of PS are poorly understood (B. Verhoven, et al., J. Exp. Med. 182:1597-1601, 1995; S.J. Martin, et al., J. Exp. Med. 182:1545-1556, 1995; D.L. Bratton, et al., J. Biol. Chem. 272:26159-26165,
20 1997.) PS exposure has been reported to be inhibited by the caspase inhibitor zVAD, implicating this family of "executioner" proteases in this process (S.J. Martin, et al., J. Biol. Chem. 271:28753-28756, 1996). In some
25 cells, inhibitors of calpain have also been reported to inhibit externalization of PS. However, the molecular link between protease activation and PS exposure remains to be elucidated. As disclosed in US Patent Application Serial No. 08/949,246 and reported in Zhou, et al. (Q.

Zhou, et al., J. Biol. Chem. 272(29):18240-18244, 1997), we recently cloned a protein, PL scramblase, that mediates the Ca²⁺-activated bi-directional transport of phospholipids between the two leaflets of a lipid bilayer membrane. This protein has been shown to transport phospholipids independent of the lipid headgroup, and we now have evidence that PL scramblase is mediating surface exposure of plasma membrane PS in all cells undergoing apoptosis (see below).

10 Role of Ceramide in Apoptosis

In recent years data have accumulated that suggest a major role for the sphingolipid ceramide as a signal transducer in the apoptotic process. It is now evident that in response to a number of stimuli, including tumor necrosis factor, ligand binding or cross-linking of Fas, serum starvation, or γ -irradiation, production of ceramide is observed (Y. Hannun, Nature 274:1855-1859, 1996; R. Kolesnick and Z. Fuks, J. Exp. Med. 181:1949-1952, 1995). Conversely, exogenous addition of ceramide to cells induces apoptosis. Activation of the so-called sphingomyelin cycle results in activation of sphingomyelinase which hydrolyses plasma membrane sphingomyelin to generate cellular ceramide. Ceramide, in turn, has emerged as a candidate intracellular mediator, affecting the activities of several enzymes, including a ceramide-activated protein phosphatase (CAPP), ceramide-activated kinases (CAPK, JNK/SAPKs), and protein kinase C (I. Herr, et al., EMBO J. 16:6200-6208,

1997). In addition to activation of sphingomyelinase, de novo synthesis of ceramide in response to an apoptotic stimulus has also been considered. The signal transduction pathways leading to activation of sphingomyelinase remain largely unresolved. It has been noted that cytokine response modifier A (CrmA), a potent inhibitor of caspases, inhibited ceramide formation and tumor necrosis factor-induced cell death, suggesting a role for proteases in the signaling and/or activation phase of apoptosis upstream of ceramide formation (G. Ddaibo, et al., J. Exp. Med. 185:481-490, 1997). As sphingomyelin is normally located primarily in the outer leaflet of the plasma membrane (A.J. Schroit and R.F.A. Zwaal, supra, 1991), it is likely that ceramide generation in the resting cell is limited by the restricted accessibility of sphingomyelin for hydrolysis by the cytoplasmic enzyme, sphingomyelinase, and that the plasma membrane phospholipid rearrangement upon induction of apoptosis results not only in surface exposure of PS, but also movement of sphingomyelin from outer to the inner leaflet of the plasma membrane. As recent evidence from our laboratory suggests that activation PL scramblase is involved in the externalization of PS in apoptotic cells and in the accelerated inward movement of the polar phospholipids phosphatidylcholine and sphingomyelin, we propose that activation of this pathway also provides additional sphingomyelin to the inner cytoplasmic leaflet of the plasma membrane as substrate

for ceramide production. As detailed below, this invention relates to compositions and methods for controlling cell and tissue susceptibility to apoptosis by modifying the transmembrane movement of the plasma membrane phospholipids between inner and outer leaflets.

Role of Aminophospholipids in Cell Clearance

In addition to the role that accelerated transbilayer movement of plasma membrane phospholipids may play in ceramide production and in transducing the apoptotic response, the concomitant *de novo* surface exposure of aminophospholipids phosphatidylserine (PS) and phosphatidylethanolamine (PE) is implicated in the activation of both complement and coagulation systems after tissue injury, and in removal of injured or apoptotic cells by the reticuloendothelial system (A.J. Schroit and R.F.A. Zwaal, Biochim Biophys. Acta 1971:313-329, 1991; V.A. Fadok, et al., J. Immunol. 148:2207-2216, 1996; R.H. Wang, et al., J. Clin. Invest. 92:1326-1335, 1993; D. Pradhan, et al., Mol. Biol. Cell 8:767-778, 1997). Although migration of these phospholipids (PL) from inner to outer plasma membrane leaflets is known to occur as an early event either in programmed cell death or in response to complement-mediated cell injury, and to be related to an elevation of intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$), until recently, little was known about the cellular constituents that participate in this process of reorganizing the topology of the plasma membrane phospholipids.

Relationship of Plasma Membrane Phospholipids to Cancer

Transformed cancer cells exhibit the capacity to proliferate through unregulated mitotic division and to resist the normal cycle of senescence and programmed cell death characteristic of most normal untransformed cells. Additionally, malignant cancer cells *in vivo* exhibit the capacity to evade detection or injury by the body's immune defenses, including cellular killing by cytotoxic T-lymphocytes, humoral killing by antibody and complement, and removal by macrophages and other phagocytic cells of the reticuloendothelial system. Whereas there is now little information as to the specific role played by the plasma membrane phospholipids in the oncogenic and malignant potential of a cell, the potential importance of the transbilayer transport of phospholipid between leaflets of the plasma membrane to the growth behavior and senescence of a cell is suggested by evidence that (1) sphingomyelin-derived ceramide participates in the intracellular signaling that results in apoptosis (supra) (2) early events in both apoptosis and immune injury include the cell surface exposure of the aminophospholipids PS and PE, (supra) and (3) cell surface PE has been shown to promote activation of the complement system including generation of the opsin C3b and the cytolytic complex, C5b-9, whereas cell surface PS has been shown to directly promote clearance through the reticuloendothelial system, by promoting cytoadhesion and phagocytosis by tissue macrophages. (R.H. Wang, et al.,

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Identity of a protein mediating bidirectional
transbilayer movement of plasma membrane phospholipids
(PL Scramblase)

Bassé, et al. and Stout, et al. recently reported
5 the purification and preliminary characterization of an
integral RBC membrane protein that, when reconstituted in
liposomes, mediates a Ca^{2+} -dependent transbilayer movement
of PL mimicking plasma membrane PL reorganization evoked
upon elevation of $[\text{Ca}^{2+}]_c$ (F. Bassé, et al., J. Biol.
10 Chem. 271:17205-17210, 1996; J.G. Stout, et al., J. Clin.
Invest. 99:2232-2238, 1997). Evidence that a protein of
similar function must also be present in platelets was
recently reported by Comfurius, et al. (P. Comfurius, et
al., Biochemistry 35:7631-7634, 1996).

15 The molecular identity of this PL scramblase protein
including cDNA and deduced protein sequence of the full-
length polypeptide was disclosed in US Patent Application
Serial No. 08/949,246 and reported in Zhou, et al. (Q.
Zhou, et al., J. Biol. Chem. 272(29):18240-18244, 1997).
20 This report also provides evidence that this same protein
mediates plasma membrane PL scramblase function in a wide
variety of human cells and tissues. Furthermore, this
report discloses cell-to-cell differences in the level of
expression of mRNA encoding PL scramblase, implying
25 tissue-specific regulation of gene expression.

Analysis of the cDNA-derived protein sequence
revealed a strongly-preferred ($p < 0.01$) inside-to-outside
orientation of the predicted 19 residue transmembrane
helix, consistent with a type II plasma membrane protein.

Most of the polypeptide (residues 1-290) thereby extends from the cytoplasmic membrane leaflet, leaving a short exoplasmic tail (residues 310-318) (Q. Zhou, et al., supra, 1997). The predicted orientation of this protein

5 is consistent with the anticipated topology of PL scramblase in the erythrocyte membrane, where lipid-mobilizing function is responsive to $[Ca^{2+}]$ only at the endofacial surface of the membrane (Q. Zhou, et al., supra, 1997; P. Williamson, et al., Biochemistry 31:6355-6360, 1992; E.F. Smeets, et al., Biochim. Biophys. Acta BioMembr. 1195:281-286, 1994; F. Bassé, et al., supra, 1996; J.G. Stout, et al., supra, 1997; P. Williamson, et al., Biochemistry 34:10448-10455, 1995; D.L. Bratton, J. Biol. Chem. 269:22517-22523, 1994).

10

15 Consistent with the divalent ion-dependence of its PL-mobilizing activity, Zhou, et al. identified a conserved EF-hand-like motif in PL scramblase that is required for the Ca^{2+} -accelerated transbilayer movement of membrane phospholipids (Zhou, et al., Biochemistry 37:2356-2360, 1998), and PL scramblase was shown to undergo a prominent conformational change upon binding Ca^{2+} (Stout, et al., Biochemistry 37:14860-14866, 1998). Human PL scramblase was shown to be thiol-esterified with palmitate at cytoplasmic cysteinyl residue(s) and

20

25 evidence provided that the normal PL-mobilizing activity of this protein is dependent upon such post-translational modification of the polypeptide with fatty acid (Zhao, et al., Biochemistry 37:6361-6366, 1998).

In addition to platelet and red blood cell, PL
scramblase activity has been observed in many other
cells, and this Ca^{2+} -induced response is thought to be
central to the rapid movement of PS and
5 phosphatidylethanolamine from inner plasma membrane
leaflet to the surface of perturbed endothelium, and a
variety of injured and apoptotic cells (R.F.A. Zwaal and
A.J. Schroit, supra, 1997). The resulting exposure of PS
at the cell surface is thought to play a key role in
10 removal of such cells by the reticuloendothelial system,
in addition to activation of both the plasma complement
and coagulation systems (R.H. Wang, et al., supra, 1993;
V.A. Fadok, et al., supra, 1992; R.F.A. Zwaal and A.J.
Schroit, supra, 1997; D. Pradhan, et al., supra, 1997).
15 Whereas the molecular mechanism(s) in each circumstance
remains unresolved, evidence for a specific platelet
membrane protein functioning to accelerate migration of
PL between membrane leaflets at increased cytosolic $[\text{Ca}^{2+}]$
has been reported (P. Comfurius, et al., supra, 1996;
20 Zhou, et al., supra, 1997), similar to the proposed role
of PL scramblase in red blood cells (F. Bassé, et al.,
supra, 1996; J.G. Stout, et al., supra, 1997). It was
thus of interest to determine whether mRNA for this
protein is expressed in nucleated cells where PL
25 scramblase-like activity has been observed.

Northern blotting with PL scramblase cDNA revealed
transcripts of ~1.6 and ~2.6 kb in all tissues and cell
lines tested (Q. Zhou, et al., supra, 1997). Some

tissue-to-tissue and cell line variability in the relative abundance of these two transcripts is apparent, the significance of which remains to be determined. Also notable was markedly reduced expression in HL-60 and the lymphoma lines Raji and MOLT-4 whereas abundant message was detected in spleen, thymus, and peripheral leukocytes. In addition to the transformed cell lines shown, mRNA for PL scramblase was also confirmed in human umbilical vein endothelial cells. Whereas these data imply that the same protein identified as mediating accelerated transbilayer flip-flop of the erythrocyte membrane PL also plays a similar role in the plasma membrane of platelets, leukocytes and other cells, actual confirmation for this role of PL scramblase awaits analysis of a cell line that is selectively deficient in this protein (Q. Zhou, et al., supra, 1997). In Scott syndrome, a bleeding disorder related to an inherited deficiency of plasma membrane PL scramblase function, erythrocytes deficient in PL scramblase activity were found to contain normal amounts of the PL scramblase protein (J.G. Stout, et al., supra, 1997). Furthermore, despite the apparent deficiency in Scott syndrome cells of endogenous PL scramblase function, when PL scramblase protein from these cells was purified and reconstituted in proteoliposomes containing exogenous PL, it exhibited normal Ca^{2+} -dependent PL-mobilizing activity (J.G. Stout, et al., supra, 1997). Consistent with these results, analysis of B lymphoblasts from a patient with Scott

syndrome by Northern blot and RT-PCR revealed normal amounts of RNA and cDNA of PL scramblase, respectively, and cDNA sequence was identical to that previously reported for PL scramblase (Q. Zhou et al., Blood 92:1707-1712, 1998). These results suggest that in addition to the known regulation by intracellular $[Ca^{2+}]$, the activity of PL scramblase in the plasma membrane is regulated by other as yet unidentified membrane or cytoplasmic component(s).

10 Relationship of PL Scramblase to Cancer

Kasukabe et al., 1997 (T. Kasukabe, et al., Blood 89:2975-2985, 1997) disclose a gene designated NOR1 that is down-regulated in transformed murine monocytes relative to amount of mRNA expressed in normal mouse cells and a 5'-truncated form of this same transcript (designated TRA1) that is over-expressed in leukemogenic mouse monocytic cell lines but not found in the non-leukemogenic monocytic cell lines. They suggest that the Tra1 gene product is associated with the leukemogenesis of monocytic Mm cell lines.

Analysis of the open reading frame predicted by the NOR1 and TRA1 cDNA sequences reveals near-identity of protein sequence with mouse or human PL scramblase in the overlapping portions of each polypeptide. The apparent identity of the full-length NOR1 gene product as the mouse homologue of human PL scramblase was recently confirmed on the basis of cDNA cloning and sequence analysis (T. Kasukabe, et al., Biochem. Biophys. Res.

Commun. 249:449-55, 1998.) As will be detailed below, the present invention relates to our discovery that the level of expression of PL scramblase influences the capacity of cells to resist complement injury or to

5 succumb to stimuli that induce programmed cell death.

The present invention also relates to the role of PL scramblase in promoting clearance of injured or apoptotic cells by the phagocytic cells of the reticuloendothelial system.

10 Needed in the art is a method for modulating the activity or level of phospholipid scramblase within a cell, organ or tissue in which one wishes either to reduce the potential for cell death and resulting clearance of beneficial cells by the scavenger
15 macrophages of the reticuloendothelial system (by *decreasing* cellular PL scramblase expression or activity) or to promote cell death and cell clearance from the body of undesirable cells (by *increasing* cellular PL
scramblase expression or activity).

20 BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

Fig. 1A is a comparison of the cDNA and deduced amino acid sequence of human PL scramblase (SEQ ID NOs:1 and 2).

Fig. 1B is a comparison of the cDNA and deduced
25 amino acid sequence of murine PL scramblase (SEQ ID NOs:3 and 4).

Fig. 2 is a bar graph illustrating immunoprecipitation of erythrocyte PL scramblase.

Fig. 3 is a graph of an activity assay of recombinant PL scramblase.

5 Fig. 4 is an immunoblot of PL scramblase in human erythrocytes and platelets.

Fig. 5 is a comparison of protein sequences of mouse and human PL scramblase (SEQ ID NOs:2 and 4).

Fig. 6 is a bar graph of PL scramblase activity as a
10 function of mutational analysis of a putative EF hand loop motif contained in human PL scramblase.

Fig. 7 graphs the Ca^{2+} dependence of mutant human PL scramblase with amino acid substitutions in putative Ca^{2+} binding site.

15 Fig. 8 is a Western blot analysis of PL scramblase protein and corresponding functional assay of PL scramblase activity in various human cell lines.

Fig. 9A and B are fluorescence micrographs of GFP-PL scramblase in transformed Raji cells. Fig. 9A depicts
20 fluorescence of cells expressing GFP; Fig. 9B depicts cells transfected with pEGFP-C2-PL scramblase plasmid and expressing GFP-PL scramblase fusion protein.

Fig. 10 is a graph showing that the level of expression of PL scramblase determines plasma membrane
25 sensitivity to intracellular calcium.

Fig. 11 is a bar graph illustrating inactivation of PL scramblase by hydroxylamine.

Fig. 12 is analysis of metabolically-labeled PL scramblase by SDS-PAGE revealing covalent incorporation of thioester-linked fatty acid.

Fig. 13 is analysis by thin layer chromatography demonstrating that PL scramblase contains covalent palmitic acid.

Fig. 14A and B is a bar graph illustrating the role of PL scramblase in PS exposure during apoptosis of B-lymphocytes. Fig. 14A compares Raji and W9 cell lines. Fig. 14B compares individual Raji cell clones that were stably transfected with either pEGFP-C2 or with pEGFP-PL scramblase and then induced to apoptosis.

Fig. 15 is a bar graph illustrating the percent apoptotic cells for Raji cells, Raji cells transfected with GFP-PL scramblase fusion protein or GFP vector as a control.

DESCRIPTION OF THE INVENTION

The present invention relates to the creation and use of reagents and methods to either inhibit or promote cell injury, cell death, and cell clearance from the body. These reagents and methods rely on the properties of phospholipid scramblase (PL scramblase), a protein that mediates Ca^{2+} -dependent transbilayer movement of plasma membrane phospholipids that are involved in the activation of the plasma complement and coagulation cascades and are substrates for cellular enzyme cascades that mediate programmed cell death.

In one embodiment, the present invention is a method for decreasing the viability of cancer cells or virus-infected cells comprising the step of increasing the amount or activity of PL scramblase in the plasma

5 membrane of the cancer cell, cancerous tissue, or virus-infected cell, thereby promoting the movement of plasma membrane phospholipids between inner and outer plasma membrane leaflets. Preferably, the increase in PL scramblase is accomplished by activating the promoter of

10 the endogenous PL scramblase gene or by delivering cDNA encoding PL scramblase, provided in a suitable targeting vector to the cell or tissue. In a preferred embodiment, cDNA encoding PL scramblase is delivered to the cell or tissue in a targeting vector, where the vector is a

15 retrovirus or adenovirus vector containing the PL scramblase cDNA.

The feasibility of this strategy is exemplified by a recent report by Der, et al. (supra, 1998) that treatment of a human fibrosarcoma cell line with interferons

20 markedly elevates expression of a number of apoptosis-related genes, including PL scramblase. Der, et al. hypothesize that the anti-tumor activity of interferons in vivo may relate to increased expression of PL scramblase in tumor cells containing interferon

25 receptors, thereby promoting movement of PS to the cell surface and accelerating clearance through the reticuloendothelial system.

In another embodiment, the present invention is a method for inducing programmed cell death (apoptosis) of a cell, preferably a mammalian cell, comprising the step of increasing the level of expression of plasma membrane PL scramblase, thereby increasing the movement of plasma membrane phospholipids between membrane leaflets, resulting in the movement of the phospholipid sphingomyelin from outer membrane leaflet to inner leaflet and the increase in production of ceramide from sphingomyelin through the action of the cytoplasmic enzyme sphingomyelinase. The resulting ceramide is known to induce activation of a cascade of cellular enzymes that ultimately lead to apoptotic cell death.

In another embodiment, the present invention is a method for promoting clearance of a cell from the body by phagocytic uptake into the reticuloendothelial system comprising the step of increasing the level of expression of plasma membrane PL scramblase, thereby increasing cell surface exposure of phosphatidylserine and phosphatidylethanolamine through the movement of these aminophospholipids from inner plasma membrane leaflet to outer plasma membrane leaflet. Cell-surface-exposed phosphatidylserine is known to directly promote adhesion and phagocytosis by macrophages and other cells of the reticuloendothelial system whereas phosphatidylethanolamine promotes cell adhesion and phagocytosis by macrophages and other cells of the reticuloendothelial system by promoting complement

activation, which opsonizes the cell surface with complement C3b.

In another embodiment, the present invention is a method of promoting the viability of a mammalian cell, both in *in vitro* cell culture and *in vivo*, comprising the step of decreasing the activity of the plasma membrane PL scramblase, thereby inhibiting movement of plasma membrane phospholipids between inner and outer membrane leaflets. The decrease in plasma membrane PL scramblase is accomplished by either decreasing expression of the endogenous PL scramblase gene or by delivering to the cell an inhibitor of the PL scramblase protein. In one embodiment, the expression of PL scramblase is inhibited by delivering to the cell antisense oligonucleotides or antisense cDNA to PL scramblase RNA. In another embodiment, a defective form of the PL scramblase protein is expressed in the cell and that mutant PL scramblase inhibits the endogenous PL scramblase. In the preferred embodiment, endogenous PL scramblase is inactivated by deacylating essential fatty acids from the protein that are required for normal PL scramblase function in the plasma membrane. By decreasing the activity of plasma membrane PL scramblase, the cell is protected due to decreased hydrolysis of plasma membrane sphingomyelin to ceramide (mediated by cellular sphingomyelinase), as well as by decreased exposure of the procoagulant and complement-activating aminophospholipids

phosphatidylserine and phosphatidylethanolamine at the cell surface.

In another embodiment, the present invention is a method of preventing the phagocytic removal and clearance of a transfused or transplanted cell by preventing surface exposure of plasma membrane phosphatidylserine by delivering to the cell a mutant phospholipid scramblase that inhibits the endogenous PL scramblase. This scramblase is preferably mutated at a site of post-translational modification, most preferably the site is selected from the group consisting of Asp²⁷³-Asp²⁸⁴, Thr¹⁶¹ and conserved cysteines of human PL scramblase or the corresponding conserved residues in mouse or other mammalian PL scramblase.

In one embodiment, a gene construct encoding a mutant phospholipid scramblase is delivered to the cell. In an alternative embodiment, the mutant protein itself is delivered.

In another embodiment the present invention is a method of diagnosing metastatic and invasive potential or growth potential of a cancer cell or cancerous tissue. This method may comprise the step of analyzing the amount of PL scramblase RNA or protein within a patient's cell sample. We predict that the presence of a comparatively low amount of PL scramblase RNA or protein will indicate the presence of cancerous tissue. The level of PL scramblase RNA may be measured by *in situ* hybridization or Northern blotting with PL scramblase cDNA or by

polymerase chain reaction using oligonucleotide primers based on sequence of human PL scramblase RNA.

Alternatively, the method may comprise the step of analyzing the sequence of a patient's PL scramblase gene,
5 RNA or protein for the presence or absence of mutations. This analysis may preferably be selected from the group consisting of restriction fragment length polymorphism, sequencing by RT-PCR, Northern blotting, Western
10 blotting, electrophoretic gels, protease digestion, and other techniques designed to analyze RNA, DNA or protein sequence.

In a preferred embodiment, the present invention involves the use of a preparation of a plasma membrane phospholipid scramblase ("PL scramblase"). It is now
15 known that PL scramblases isolated from various species have sequence variations. By "PL scramblase" we include these variants as well as the proteins as described in U.S. Serial No. 08/790,186, Serial No. 08/949,246, Zhao,
et al. (J. Biol. Chem. 272(29):18240-18244, 1997), and
20 Kasukabe, et al., (Biochem. Biophys. Res. Comm. 249:449-55, 1998) and genes or cDNAs encoding these proteins.

Preferably, the protein is approximately 35-37 kD as measured on a 12.5% SDS polyacrylamide gel under reducing conditions. In a most preferred form of this invention,
25 the preparation is a human or a mouse PL scramblase.

In one preferred embodiment of the present invention, the PL scramblase comprises SEQ ID NO:2, representing human PL scramblase, possibly with conservative or functionally equivalent substitutions.

5 In the most preferred embodiment of the present invention, the PL scramblase has been modified by the action of mammalian cellular enzymes to covalently incorporate phosphorus at one or more Thr, Ser, or Tyr residues or a fatty acid, preferably palmitate, at a
10 cysteine residue.

The present invention may also use a murine cell protein, wherein the protein is a plasma membrane phospholipid scramblase, preferably wherein the protein is approximately 35 kD as measured on a 12.5%
15 SDSpolyacrylamide gel under reducing conditions.

In another preferred embodiment of the present invention, the murine PL scramblase has been modified by the action of mammalian cellular enzymes to covalently incorporate phosphorus at one or more Thr, Ser, or Tyr
20 residues, and a fatty acid, preferably palmitate, at a cysteine residue.

The present invention may employ an inhibitor of the PL scramblase activity of PL scramblase. This inhibitor may be an antisense nucleotide derived from the DNA
25 sequence of PL scramblase. In another embodiment, the inhibitor is a peptide sequence that is a competitive inhibitor of PL scramblase activity. In another

embodiment, the inhibitor is an antibody, preferably a monoclonal antibody, raised against PL scramblase.

In another embodiment, the inhibitor works by modifying or inhibiting the post-translational
5 modifications of the PL scramblase that are disclosed below in the Examples. For example, analysis of the primary PL scramblase sequence reveals a potential site of phosphorylation by protein kinase C or other cellular kinase (Thr¹⁶¹), potential sites for acylation by fatty
10 acid, and a potential binding site for Ca²⁺ ion provided by an EF-hand-like loop spanning residues Asp²⁷³-Asp²⁸⁴. These residues and motifs are conserved in the mouse PL scramblase.

In another embodiment, the inhibitor is a compound
15 that prevents thioacylation of the protein.

In another embodiment, a mutant phospholipid scramblase is provided in which cysteine residues have been replaced by alanine or other non-conservative substitution.

20 The present invention is also a method for promoting the viability and circulating lifetime of *in vitro* stored leukocytes, lymphocytes, platelets, or red blood cells. This method comprises the steps of adding an inhibitor of PL scramblase activity to the stored blood cells.

25 The present invention is also a method for prolonging survival of transplanted cells, tissues, or organs, comprising the step of adding an inhibitor of PL scramblase activity to an organ perfusate or cell storage

medium during *in vitro* organ storage. The present invention is also a method for prolonging the survival of transplanted cells, tissues, and organs by genetically engineering the cells to be transplanted so as to reduce
5 their expression of plasma membrane PL scramblase in order to reduce exposure of PS and other thrombogenic and pro-inflammatory phospholipids at the plasma membrane surface. In one embodiment of the present invention, the transplanted cells to be treated are hematopoietic stem
10 cells to be used to repopulate the bone marrow by transfusion into a patient.

The present invention is also a method for diagnosing the survivability, metastatic, and invasive potential of a cancer cell or cancerous tissue or virus-
15 infected cell by quantifying the level of cellular expression of PL scramblase and by detecting deletion or loss of function mutations in PL scramblase. For example, this quantitation may take the form of immunoblotting using an antibody to PL scramblase, an
20 ELISA assay using an antibody to PL scramblase, flow cytometric analysis of the binding of monoclonal antibody reactive against the predicted extracellular domain of PL scramblase (residues Ser³¹⁰ Trp³¹⁸ of sequence disclosed in SEQ ID NO:2 or the equivalent residue in the conserved
25 region of another PL scramblase) or using oligonucleotides derived from PL scramblase cDNA and the polymerase chain reaction. In one method, Northern blotting or *in situ* hybridization is performed using cDNA

of human PL scramblase as probe. In another method of the present invention, mutations in the PL scramblase gene locus are detected by DNA sequencing of the cDNA product of the polymerase chain reaction and by analysis of restriction length polymorphisms. In another method of the present invention, the quantitation is performed by isolating PL scramblase from tissue biopsy or a patient blood sample, measuring the amount of PL scramblase isolated and comparing the measurement with a control sample. The measurement may be by isolating PL scramblase from a patient tissue biopsy or blood sample and measuring via densitometry the amount of PL scramblase protein electrophoresed in a stained electrophoretic gel.

15

EXAMPLES

EXAMPLE 1: Cloning of Murine PL scramblase and Identity of a Conserved Motif in Phospholipid Scramblase that is Required for Accelerated Transbilayer Movement of Membrane Phospholipids by Ca^{2+} (Zhou, et al., Biochemistry 37:2356-2360, 1998).

20

A. Summary

Accelerated transbilayer movement of plasma membrane phospholipids (PL) upon elevation of Ca^{2+} in the cytosol plays a central role in the initiation of plasma clotting and in phagocytic clearance of injured or apoptotic cells. We recently identified a human erythrocyte membrane protein that induces rapid transbilayer movement

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of PL at elevated Ca^{2+} , and presented evidence that this PL scramblase is expressed in a variety of other cells and tissues where transbilayer movement of plasma membrane PL is promoted by intracellular Ca^{2+} (Q. Zhou, et al., J. Biol. Chem. 272:18240-18244, 1997). We have now cloned murine PL scramblase for comparison to the human polypeptide (Fig. 1B): Both human and murine PL scramblase are acidic proteins ($\text{pI}=4.9$) with a predicted inside-outside (type 2) transmembrane segment at the carboxyl-terminus (Fig. 5). Whereas human PL scramblase (318 AA) terminates in a short exoplasmic tail, murine PL scramblase (307 AA) terminates in the predicted membrane-inserted segment. The aligned polypeptide sequences reveal 65% overall identity, including near identity through 12 residues of an apparent Ca^{2+} binding motif (D[A/S]DNFGIQFPLD) spanning residues 273-284 (human, SEQ ID NO:2) and 271-282 (murine, SEQ ID NO:4), respectively (Fig. 5). This conserved sequence in the cytoplasmic domain of PL scramblase shows similarity to Ca^{2+} -binding loop motifs previously identified in known EF-hand structures. Recombinant murine and human PL scramblase were each expressed in *E. coli* and incorporated into proteoliposomes. Measurement of transbilayer movement of NBD-labeled PL confirmed that both proteins catalyzed Ca^{2+} -dependent PL flip/flop similar to that observed for the action of Ca^{2+} at the cytoplasmic face of plasma membranes. Mutation of residues within the putative EF hand loop of human PL scramblase resulted in loss of its

PL mobilizing function, suggesting that these residues directly participate in the Ca^{2+} induced active conformation of the polypeptide.

B. Experimental Procedures

5 Abbreviations used: PL, phospholipids(s); PC, phosphatidylcholine; PS, phosphatidylserine; NBD-PC, 1-oleoyl-2-[6(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl-sn-glycero-3-phosphocholine; EST, expressed sequence tag; MBP, maltose binding protein;
10 PAGE, polyacrylamide gel electrophoresis; bp, base pair(s); PCR; polymerase chain reaction.

Materials: Mouse fibroblast 5'-stretch plus cDNA library and KlenTaq polymerase were obtained from CLONTECH Laboratories. Expressed sequence Tag (EST)
15 clone with GenBank™ accession number gb AA110551 was from American Type Culture Collection (ATCC 977052). α - ^{32}P -dCTP was purchased from Dupont. Random Primed DNA Labeling Kit was from Boehringer Mannheim. Hybond-N Nylon membrane was from Amersham. Expression vector
20 pMAL-C2, amylose resin and all restriction enzymes were from New England Biolabs. Wizard Kit was from Promega. Qiagen Lambda Kit was from Qiagen. Egg yolk phosphatidylcholine (PC), brain phosphatidylserine (PS) and 1-oleoyl-2-[6(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl-sn-glycero-3-phosphocholine (NBD-PC)
25 were obtained from Avanti Polar Lipids. Factor Xa was from Haematologic Technologies, and Bio-Beads SM-2 were from BioRad. N-octyl- β -D-glucopyranoside and Glu-Gly-Arg

chloromethyl ketone were from Calbiochem. Sodium dithionite (Sigma) was freshly dissolved in 1 M Tris, pH 10, at a concentration of 1 M.

Labeling of DNA Probe: The DNA insert of EST clone
5 gb AA110551 was released by digestion with *EcoRI* and *ApalI* and purified by Wizard Kit. Four micrograms of purified DNA were labeled with 1 mCi of α - ^{32}P -dCTP. The specific radioactivity of the probe was 3.9×10^8 dpm/ μg DNA.

10 Isolation of Mouse PL Scramblase cDNA by Plaque

Hybridization: *E. coli* strain Y1090r was transformed by mouse fibroblast cDNA library (6×10^5 pfu) and poured onto 30 plates (15 cm diameter, 20,000 pfu per plate). Plaques were lifted onto Hybond-N Nylon
15 membranes. After denaturation, neutralization and UV-cross-linking, the membranes were first prehybridized in a solution composed of 5X Denhardt, 5X SSC, 1% SDS, and 200 $\mu\text{g/ml}$ herring sperm DNA for 3 hours at 68°C , and then hybridized in the same solution containing 5 ng/ml ^{32}P -
20 labeled probe for 16 hours at 68°C . The membranes were washed once with 2X SSC, 0.1% SDS, then three times with 0.1 X SSC, 0.1% SDS for 20 minutes at 65°C , and exposed to X-ray film. Secondary plaque lifts and hybridization were carried out on 8 positive plaques at a density of
25 about 100 plaques/plate. Single positive and well isolated plaques were picked and amplified. λ DNA was purified with Qiagen Lambda Maxi Kit.

DNA Sequencing. DNA was sequenced on an ABI DNA Sequencer Model 373 Stretch (Applied Biosystems) using PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Perkin Elmer).

5 Cloning of Mouse PL Scramblase into pMAL-C2

Expression Vector. In order to express mouse PL scramblase as a fusion protein with maltose binding protein (MBP), cDNA encoding mouse PL scramblase was cloned into pMAL-C2 expression vector. PCR was performed
10 on a mouse scramblase clone using the primers 5'TCA GAA TTC GGA TCC ATG GAG GCT CCT CGC TCA GGA AC³' (SEQ ID NO:8) with an *EcoRI* site before the ATG start codon and 5'GCT TGC CTG CAG GTC GAC CTA CAC ACA GCC TTC AAA AAA CAT G³' (SEQ ID NO:9) with a *SalI* site after the stop codon.
15 KlenTaq polymerase was used to ensure high fidelity amplification. The PCR product was digested with *EcoRI* and *SalI*, isolated by electrophoresis, and cloned into pMAL-C2 immediately 3' of MBP. *E. coli* strain TB1 was transformed, and sequence of the cDNA insert of plasmid
20 from a single colony was confirmed.

Expression and Purification of Mouse PL Scramblase-MBP Fusion Protein: Mouse PL scramblase was expressed as fusion protein with MBP in *E. coli* TB1 and purified on amylose resin as previously described for human PL
25 scramblase (Q. Zhou, et al., J. Biol. Chem. 272:18240-18244, 1997). The purified fusion protein was centrifuged at 106,000 x g for 1 hour at 4°C to remove aggregated protein.

Reconstitution and Functional Activity of PL

Scramblase: Reconstitution, removal of MBP, and functional assay of PL scramblase were performed as previously described (F. Bassé, *et al.*, *J. Biol. Chem.* 271:17205-17210, 1996; Q. Zhou, *et al.*, *supra*, 1997; J.G. Stout, *et al.*, *J. Clin. Invest.* 99:2232-2238, 1997). Routinely, 420 pmoles of protein were reconstituted with 1 μ mol of PL. To remove MBP, proteoliposomes were incubated 3 hours at room temperature with 1/40 (w/w) factor Xa. The digest was terminated by addition of 100 μ M Glu-Gly-Arg chloromethyl ketone. Proteoliposomes labeled with NBD-PC were incubated for 2 hours at 37°C in Tris buffer in the presence or absence of CaCl_2 as indicated in figure legends and diluted 25-fold in Tris buffer containing 4 mM EGTA. Initial fluorescence was recorded (SLM Aminco 8000 spectrofluorimeter; excitation at 470 nm, emission at 532 nm), 20 mM dithionite was added, and the fluorescence was continuously monitored for a total of 120 seconds. Scramblase activity was calculated according to the difference in non-quenchable fluorescence observed in presence vs absence of CaCl_2 . Ionized $[\text{Ca}^{2+}]$ was calculated using FreeCal version 4.0 software (generously provided by Dr. Lawrence F. Brass, University of Pennsylvania, Philadelphia, PA).

Protein Concentrations: Protein concentrations were estimated based upon optical density at 280 nm, using extinction coefficients ($\text{M}^{-1}\text{cm}^{-1}$) of 39,000 (PL

scramblase), 64,500 (MBP), and 105,000 (PL scramblase-MBP fusion protein).

Mutagenesis of PL Scramblase: Human PL scramblase amino acid residues in EF-hand Ca^{2+} -binding motif at positions of Asp²⁷³, Asp²⁷⁵, Phe²⁷⁷, Ile²⁷⁹, Phe²⁸¹ and Asp²⁸⁴ were mutated to Ala with oligonucleotide-directed mutagenesis by two rounds of PCR. PL scramblase-pMAL-C2 was selected as template, and the first round of PCR was performed with pairs of a complementary oligonucleotide primer containing the point mutation plus a primer complementary to a site near the ATG initial codon or TAG stop codon. PCR products were purified by Wizard kit. Full-length mutated PL scramblase cDNA was obtained by overlapping PCR and cloned back into pMAL-C2 vector. After confirmation of correct DNA sequence the mutants were recombinantly expressed in *E. coli* as described above and analyzed by SDS-PAGE.

C. Results and Discussion

Isolation of cDNA of Mouse PL Scramblase. Murine EST clones in GenBank containing putative PL scramblase sequence were identified by a Blast homology search using the human PL scramblase cDNA. Among several clones exhibiting significant homology, a 403 bp Stratagene mouse kidney clone (gb accession number AA110551) with 79% nucleotide sequence identity to human PL scramblase was selected and this clone was used to probe a mouse fibroblast cDNA library. Eight positive clones were identified after two rounds of plaque hybridization. Two

of the eight clones were sequenced yielding 1354 bp and 1529 bp, respectively. Alignment revealed 1261 bp of overlapping sequence that spanned an open reading frame of 921 bp and specified a total of 1622 bp of unique cDNA sequence (SEQ ID NO:3).

SEQ ID NO:4 represents the open reading frame of the translated sequence of SEQ ID NO:3 (see Fig. 1B). The deduced mouse PL scramblase cDNA encodes a 307 residue protein with a molecular weight of 33.9 kDa and a theoretical pI = 4.9, similar to values obtained for the human protein (318 residues, 35.1 kDa; pI=4.9; ref. (Q. Zhou, et al., supra, 1997)). The overall identity of the mouse and human PL scramblase is 64.8%, with the most divergent sequence generally contained in the N-terminal portion of the polypeptide (Fig. 5). Fig. 5 depicts the alignment of protein sequences of mouse and human PL scramblase. Alignment between mouse (MUR) and human (HUM) PL scramblase was performed by FASTA program using the Smith-Waterman algorithm. (W.R. Pearson and D.J. Lipman, Proc. Natl. Acad. Sci. USA 85:2444-2448, 1988) Sequence of human PL scramblase is contained in GenBank™ accession number AF008445. Amino acid identities (:) or similarities (.) between the two sequences are indicated. Also indicated are the residues comprising a predicted inside-out transmembrane domain (MUR 289-307, HUM 291-309; double underline), and the 12 residues of the acidic loop of a putative EF-hand (MUR 271-282, HUM 273-284; single underline).

In both proteins, a single 19 residue transmembrane helix is predicted at the carboxyl terminus, exhibiting a strongly preferred inside-to-outside orientation. Whereas the mouse protein terminates immediately after this conserved transmembrane helix, the human PL scramblase contains an additional nine residues, implying that the short exoplasmic peptide in human PL scramblase is non-essential to function. Homology motifs conserved in both proteins include a potential site for protein kinase C phosphorylation (Thr¹⁵⁹ in mouse, Thr¹⁶¹ in human) and a potential Ca²⁺-binding EF-hand loop motif adjacent to the transmembrane helix (residue Asp²⁷¹ to Asp²⁸² in mouse and residues Asp²⁷³ to Asp²⁸⁴ in human). The cytoplasmic orientation of this protein and the proximity of this putative Ca²⁺-binding domain to the segment of polypeptide that is inserted into the plasma membrane are consistent with the proposed activity of this protein *in situ*, where Ca²⁺ acting directly at the endofacial membrane surface is known to initiate the rapid transbilayer movement of plasma membrane PL (P. Williamson, *et al.*, Biochemistry 31:6355-6360, 1992; R.F.A. Zwaal and A.J. Schroit, Blood 89:1121-1132, 1997; F. Bassé, *et al.*, J. Biol. Chem. 271:17205-17210, 1996; D.L. Bratton, J. Biol. Chem. 269:22517-22523, 1994; B. Verhoven, *et al.*, Biochim. Biophys. Acta 1104:1523, 1992).

Functional Activity of Recombinant Mouse PL

Scramblase. In order to confirm that the cDNA identified

as mouse PL scramblase encodes a protein of similar function to that identified in human, the human and mouse proteins were each expressed in *E. coli*, purified, and reconstituted in proteoliposomes for measurement of PL mobilizing activity. Mouse or human PL scramblase-MBP fusion protein (420 pmoles) was reconstituted into PC/PS liposomes (1 μ mol total PL), respectively, MBP was removed by digestion of the proteoliposomes with factor Xa, and PL scramblase activity was determined as described under "Experimental Procedures" and plotted as a function of external free $[Ca^{2+}]$. The results of this experiment indicate that recombinant mouse PL scramblase mediated a Ca^{2+} -dependent transbilayer movement of membrane PL with a specific activity and affinity for Ca^{2+} indistinguishable from that observed for the recombinant human protein.

Mutational Analysis of a Putative Conserved EF-Hand Motif. As noted above, the deduced protein sequence of mouse and human PL scramblase reveals an extensive segment of highly conserved sequence extending through residue Glu³⁰⁶ (in human; corresponding to Glu³⁰⁴ in mouse; Fig. 5). The predicted secondary structure through this portion of the protein reveals that it contains two short alpha-helical segments near the C-terminus that are separated by a 12-residue acidic loop. In both proteins (human and mouse), the C-terminal alpha helix represents a predicted transmembrane segment with a strongly-preferred inside-to-outside orientation, whereas sequence

contained within the adjacent 12-residue acidic loop conforms in-part to a consensus sequence that is characteristic of an EF-hand Ca^{2+} -binding loop motif (S. Nakayama and R.H. Kretsinger, Annu. Rev. Biophys. Biomol. Struct. 23:473-507, 1994). In this motif, residues in positions 1, 3, 5, 7, 9 and 12 of the loop contribute to octahedral coordination of the Ca^{2+} ion, with the residues in position 1 [Asp], 3 [Asp, Asn, or Ser] and 12 [Asp or Glu] being those most highly conserved.

10 In order to gain insight into whether this segment of the protein might be directly involved in the Ca^{2+} -dependent reorganization of membrane PL mediated by PL scramblase, we expressed mutant human PL scramblase with Ala substitutions at positions corresponding to residues
15 1 (Asp^{273}), 3 (Asp^{275}), 5 (Phe^{277}), 7 (Ile^{279}), 9 (Phe^{281}), and 12 (Asp^{284}) of this putative 12 residue EF-hand loop.

Fig. 6 illustrates PL scramblase activity as a function of mutational analysis of putative EF hand loop motif contained in human PL scramblase. Wild-type (WT)
20 and mutant constructs of human PL scramblase were expressed as fusion proteins with MBP in *E. coli*, purified, and reconstituted in proteoliposomes. After release of MBP by incubation with factor Xa, PL scramblase activity was assessed (see "Experimental
25 Procedures"). For each mutant construct, the residues in human PL scramblase that were replaced by Ala are indicated on the *abscissa*. PL scramblase activity (*ordinate*) was measured in presence of 2 mM CaCl_2 , and in

each case was normalized to the activity of WT human PL
scramblase ($11.76 \pm 0.44\%$ of total NBD-PC flipped), with
correction for the non-specific transbilayer movement of
NBD-PC ($0.20 \pm 0.08\%$ of total NBD-PC flipped) measured in
5 PL vesicles lacking added protein. Error bars indicate
mean \pm SD of three independent measurements performed
with each mutant construct. Fig. 6 illustrates the data
of single experiment, representative of two separate
experiments so performed.

10 As illustrated by Fig. 6, Ala substitution at any of
these positions reduced PL scramblase function, with
mutation at Asp²⁷⁵ resulting in complete inactivation of
the Ca²⁺-dependent response. In those mutant polypeptides
showing partial retention of activity, reduced response
15 to Ca²⁺ was related in-part to an apparent reduction in
avidity for Ca²⁺ (Fig. 7).

Fig. 7 illustrates the Ca²⁺-dependence of mutant
human PL scramblase. PL scramblase activity of wild-type
(WT) and selected mutant constructs of Fig. 6 was
20 determined as described in "Experimental Procedures" and
plotted as a function of external free [Ca²⁺]: WT(●);
Asp²⁷³(□); Phe²⁷⁷(Δ); Ile²⁷⁹(◇); Phe²⁸¹(○); Asp²⁸⁴(∇). The
data are corrected for non-specific transbilayer
migration of NBD-PC in the absence of free [Ca²⁺]. Data
25 of single experiment. The results described in Fig. 7
suggest that residues contained in the putative EF-hand
loop spanning Asp²⁷³-Asp²⁸⁴ are critical to the function of
PL scramblase, presumably for coordination of Ca²⁺ as

required to induce the PL transporting state of the protein. It remains to be determined what conformational changes are induced in the polypeptide in the presence of Ca^{2+} , including potential reorientation of helical segments flanking the putative Ca^{2+} binding loop, that might contribute to the accelerated transbilayer movement of membrane phospholipids.

EXAMPLE 2: Inactivation of Human PL Scramblase by Treatment With the Thiolester Cleaving Reagent, Hydroxylamine (Zhao, et al., Biochemistry 37:6361-6366, 1998).

A. Summary

Incubation of human erythrocyte PL scramblase with hydroxylamine under conditions known to favor hydrolysis of protein cysteinyl-fatty acyl bonds was found to cause near complete loss of PL scramblase's function in promoting movement of PL between membrane leaflets. These data suggest that for normal activity, the PL scramblase polypeptide requires post translational modification through addition of a thiolester-linked fatty acid. Furthermore, these data imply that methods that either prevent cellular acylation of the polypeptide, or that cleave cysteinyl thiolester linkages, will effectively inhibit endogenous PL scramblase activity.

B. Methods

Protein purification. PL scramblase was purified from human erythrocyte ghost membranes as previously

described (F. Bassé, et al., supra, 1996; J.G. Stout, et al., supra, 1997).

Treatment with Hydroxylamine. PL scramblase was incubated 1 hour at room temperature in 1 M hydroxylamine, 25 mM octylglucoside, 1 M Tris-HCl at pH 7.4. Match control samples of the protein were identically incubated under these conditions, omitting hydroxylamine. After incubation, samples were dialyzed and reconstituted into PL proteoliposomes for assay of PL scramblase activity.

Membrane reconstitution and assay. PL scramblase was reconstituted into proteliposomes and activity determined as previously described (F. Bassé, et al., supra, 1996; J.G. Stout, et al., supra, 1997).

15 C. Results and Discussion

As shown in Fig. 11, incubation with hydroxylamine resulted in nearly complete inactivation of PL scramblase. Fig. 11 illustrates inactivation of PL scramblase function by treatment with hydroxylamine. The figure shows loss of functional activity of human erythrocyte PL scramblase due to treatment with the thioester cleaving reagent hydroxylamine. Identical aliquots of human erythrocyte PL scramblase in 50 mM octylglucoside were incubated 1 hour room temperature in either 1 M hydroxylamine, pH 7.2 (solid bar) or 1 M Tris-HCl, pH 7.2 (open bar). After treatment, each sample was reconstituted into proteoliposomes and PL scramblase activity measured using NBD-phosphatidylcholine as probe

according to methods previously described (Basse, et al.,
supra, 1996; Stout, et al., supra, 1997; Zhou, et al.,
supra, 1997). Error bars denote mean \pm SD of seven
independent experiments. Significance by paired
5 students' t-test, $p < 10^{-6}$. (See Methods, Example 3.)

Because the conditions of incubation (neutral pH)
were chosen to favor specific cleavage of cysteinyl
thioester bonds without disulfide bond reduction, these
results imply an essential thioester linkage within the
10 protein. In a membrane-associated protein with
cytoplasmic Cys residues, such as found in erythrocyte PL
scramblase, this thiolester bond is normally provided by
palmitic acid in ester linkage to one or more cysteinyl
thiols. (H. Schroeder, et al., J. Cell Biol. 134:647-660,
15 1996; M. Stauffenbiel, J. Biol. Chem. 263:13615-13622,
1988; C.A. Wilcox, et al., Biochemistry 26:1029-1036,
1987). Whereas the possibility of disulfide reduction by
hydroxylamine cannot be excluded, it is important to note
that (1) virtually all cysteine residues in PL scramblase
20 are normally exposed to cytoplasmic reducing agents such
as glutathione, and disulfide bond formation is therefore
not anticipated and (2) The absence of any functionally-
important disulfide bonds in PL scramblase can be assumed
based on the retention of normal PL scramblase activity
25 when the protein was incubated in various reducing
agents, including dithiothreitol (F. Bassé, et al.,
supra, 1996; J.G. Stout, et al., supra, 1997). Thus
these data suggest that PL scramblase polypeptide

requires post-translational modification through addition of a thiolester-linked fatty acid for its normal function in the plasma membrane. Furthermore, these data imply that reagents that either prevent cellular acylation of the polypeptide, or, reagents that cleave cysteinyl thiolester linkages, will effectively inhibit endogenous PL scramblase activity.

The presence of covalently-linked palmitic acid in PL scramblase was directly confirmed by metabolic labeling of the protein with [^3H]-palmitate. Human Raji cells, which we have shown to contain low amounts of endogenous PL scramblase, were transformed with plasmid encoding the human PL scramblase as a fusion protein with green fluorescent protein (GFP), and metabolically labeled with [^3H]-palmitate. Immunoprecipitation with antibody to GFP revealed the covalent incorporation of [^3H] in GFP-PL scramblase fusion protein, whereas no radioactivity was associated with GFP (Fig. 12).

Fig. 12 illustrates that metabolic labeling of PL scramblase with [^3H]-palmitate reveals covalent thioester-linked fatty acid. The presence of cysteinyl-linked palmitate in PL scramblase was confirmed by metabolic labeling of pEGFP-PL scramblase-transfected Raji cells (GFP-PLS) or identically-treated vector-only controls (GFP) with exogenous [^3H]-palmitate. Cells were incubated (10^7 cell/ml, 1 hour, 37°C) with $200\ \mu\text{Ci/ml}$ [^3H]-palmitate (Dupont NEN, spec. act. $60\ \text{Ci/mmol}$) in RPMI-1640 containing 20% dialyzed fetal bovine serum and 5 mM

sodium pyruvate. Cells were then harvested, washed and membrane proteins (10^8 cells/ml) extracted into 1% triton X100, 5 mM EDTA, 20 mM leupeptin, 20 mM M-ethylmaleimide, 10 mM benzamidine, 1 mM phenylmethanesulfonyl fluoride in tris-buffered saline, pH 7.4. Insoluble material was removed by centrifugation and the supernatants precleared by incubation with 10 μ g/ml normal rabbit IgG and 30 μ l protein A sepharose (Pharmacia). The GFP and GFP-PLS proteins were then immunoprecipitated overnight with 50 μ g/ml affinity purified rabbit anti-GFP (Clontech) and staph A sepharose. The beads were exhaustively washed with 1% triton X100 and GFP vector control (Lanes 1 and 2) and the GFP-PL scramble fusion protein (Lanes 3 and 4) each eluted from staph A sepharose into 5% SDS-PAGE sample buffer. Each sample was divided and incubated 1 hour with either 1 M hydroxylamine, pH 7.2 (+) or 1 M Tris, pH 7.2 (-) and then subjected to polyacrylamide gel electrophoresis under non-reducing conditions (4-20% polyacrylamide; NOVEX). UPPER PANEL: Western blotting was performed with murine monoclonal antibody against human PL scramble (anti-PLS). MIDDLE PANEL: Western blotting performed with murine monoclonal antibody against green fluorescent protein (anti-GFP; Clontech)). LOWER PANEL shows fluorogram of radioactivity from ^3H . Fluorography of the SDS-PAGE gel was performed after fixation in 50% ethanol, 5% acetic acid, and the washed gel soaked in Enlightning scintillant (Dupont-NEN) before drying and exposure to preflashed Kodak X-AR5 film (-

80°C, 4 days). Note radioactivity in GFP-PL scramblase (see autoradiography of ~65 kDa protein band, lane 4) that is released from GFP-PL scramblase by treatment with hydroxylamine (see radioactivity at dye front, lane 3).

5 Data of single experiment, representative of two independent experiments.

The identity of the covalently-bound [³H] in PL scramblase as a thioester-linked [³H]-palmitate was confirmed by hydroxylamine cleavage and by thin layer
10 chromatography (TLC) of the liberated fatty acid (Fig. 13).

Fig.13 illustrates that TLC analysis of [³H]-fatty acid in PL scramblase. The GFP-PLS protein band at ~65 kDa of the previous figure was sliced from the wet gel
15 and the gel soaked in 30% methanol, 10% acetic acid, washed exhaustively with 50% methanol, and then lyophilized. To liberate covalently-bound fatty acids, the dried gel was incubated overnight in 1.5N NaOH. Following acidification with HCl (pH<2), the released
20 fatty acids (1 ml) were extracted by addition of 3.75 ml chloroform:methanol (1:2 v:v). To separate the fatty acids into a chloroform phase, 1.25 ml chloroform and 1.25 ml H₂O were then added, and the bottom layer recovered and dried under nitrogen. The dried pellet was
25 taken up in chloroform:methanol (2:1, v:v) and spotted onto KC18 silica gel (Whatman 4801-425). The chromatograph was developed in acetonitrile:acetic acid (9:1, v:v) and air dried. The dried plate was sprayed

with Enhance (Dupont-NEN) and radioactivity was detected by fluorography on preflashed Kodak XAR-5 film (-80°C, 4 days). Radioactivity liberated from GFP-PLS (lane 2) is compared to matched gel slice from GFP vector control (lane 3), using [³H]-palmitate as standard (standards applied in lanes 1 and 4). All samples were spotted at position OR; SF indicates position of the solvent front. Data of single experiment.

Thus, PL scramblase is palmitoylated at one or more cysteine residues. We also have evidence that such acylation is required for normal function of PL scramblase protein. As noted above, removal of palmitate through hydrolysis of thioester bonds with hydroxylamine was found to abolish PL scramblase's phospholipid mobilizing function (Fig. 11).

EXAMPLE 3: Plasma Membrane Expression of Phospholipid Scramblase Regulates Ca²⁺ Induced Movement of Phosphatidylserine to the Cell Surface: Alteration of Phosphatidylserine Exposure In Human Lymphoblasts Through Stable Transfection with PL Scramblase cDNA.

A. Summary

In order to determine whether PL scramblase is responsible for the rapid movement of PS from inner-to-outer plasma membrane leaflets in other cells exposed to elevated cytosolic [Ca²⁺]_c, we analyzed how induced movement of PS to the surface related to cellular content of PL scramblase. Exposure to Ca²⁺ ionophore A23187 resulted in rapid PS exposure in those cells high in PL

scramblase (K-562, HEL, 293T, and EBV-transformed lymphocytes), whereas this response was markedly attenuated in cells with low amounts of the protein (Raji, MOLT-4, HL-60). To confirm this apparent

5 correlation between PL scramblase expression and PS egress at elevated $[Ca^{2+}]_c$, Raji cells were transfected with PL scramblase cDNA in pEGFP-C2, and stable transformants expressing various amounts of rGFP-PL

10 scramblase fusion protein obtained. Clones expressing rGFP-PL scramblase showed plasma membrane-localized fluorescence and elevated PL scramblase antigen whereas clones expressing rGFP alone (transfected with pEGFP-C2 without insert) showed only cytoplasmic fluorescence and served as controls. In absence of ionophore, expression

15 of rGFP-PL scramblase had no effect on cell viability or background PS exposure. In response to A23187, clones expressing GFP-PL scramblase exhibited markedly accelerated movement of PS to the cell surface when compared to A23187-treated clones expressing GFP with PS

20 movement to the cell surface increasing with amount of rGFP-PL scramblase expressed. These data indicate that transfection with PL scramblase cDNA promotes $[Ca^{2+}]_c$ -dependent movement of PS to the cell surface and suggest that this protein normally mediates redistribution of

25 plasma membrane phospholipids in activated, injured, or apoptotic cells exposed to elevated $[Ca^{2+}]_c$.

B. Materials and Methods

Materials. All restriction enzymes were from New England BioLabs, Inc. (Beverly, MA). KlenTaq polymerase and pEGFP-C2 vector were from CLONTECH Laboratories (Palo Alto, CA). Bovine coagulation factor Va (FVa), factor Xa (FXa), prothrombin and dansylarginine-N-(3-ethyl-1,5-pentanedyl)amide were from Haematologic Technologies, Inc. (Essex Junction, VT). Chromogenic thrombin substrate S2238 was from DiaPharma Group, Inc. (Franklin, OH). Human α -thrombin was a generous gift from Dr. John W. Fenton (Albany, NY). OPTI-MEM and geneticin were from Life Technologies (Gaithersburg, MD). Fetal bovine serum, RPMI 1640, Cell Dissociation Solution, Hank's Balanced Salt Solution (HBSS), Protein A Sepharose-CL4B, leupeptin, and BSA were from Sigma Chemical Co. (St. Louis, MO). UltraLink Iodoacetyl resin and SuperSignal ULTRA Chemiluminescence Kit were from Pierce Chemical Co. (Rockford, IL). All other chemicals were of reagent grade.

Cell culture: Human cancer cell lines erythroleukemic HEL, promyelocytic leukemia HL-60, chronic myelogenous leukemia K562, lymphoblastic leukemia MOLT-4, acute T-cell leukemia Jurkat, Burkitt's lymphoma Raji, and megakaryocytic DAMI were from American Type Culture Collection (Rockville, MD) and cultured in RPMI 1640 containing 10% fetal bovine serum. EBV-transformed cell line W9 established from peripheral B-lymphocytes of

a normal donor was maintained as previously described (H. Kojima, et al., 1994).

Antibodies: Anti-GFP: murine monoclonal antibody against green fluorescent protein (GFP) was from CLONTECH Laboratories. Anti-FVa: murine monoclonal antibody V237 reactive against human or bovine factor Va light chain was the generous gift of Dr. Charles T. Esmon (Oklahoma Medical Research Fndn, Oklahoma City, OK). Anti-PL Scramblase-E306-W318: Rabbit antibody raised against the carboxyl terminal peptide sequence E306-W318 of human PL
10
scramblase has previously been described (Q. Zhou, et al., supra, 1997). The IgG fraction was isolated on protein A-Sepharose-CL4B and the peptide-reactive antibody purified by affinity chromatography on peptide
15
[Cys]-ESTGSQEQKSGVW (SEQ ID NO:5) coupled to UltraLink Iodoacetyl resin.

Plasmid Construction: Human PL scramblase cDNA insert was released from plasmid pMAL-C2-PL scramblase (Q. Zhou, et al., supra, 1997) by double cutting with
20
EcoRI and *SallI*, respectively, and then ligated into pEGFP-C2 vector using the same restriction site. The pEGFP-C2-PL scramblase plasmid was amplified from single clones in *E. coli* strain Top 10, and the orientation and reading frame of the insert confirmed by sequencing on an
25
ABI DNA Sequencer Model 373 Stretch (Perkin Elmer-Applied Biosystems, Foster City, CA) using PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit.

Transfection of Raji cells with pEGFP-PL scramblase.

1.6 x 10⁷ Raji cells were electroporated with 160 µg plasmid DNA (pEGFP-C2-PL scramblase or pEGFP-C2) in a total volume of 0.8 ml OPTI-MEM, using Gene Pulse
5 Electroporator (Bio-Rad Laboratories, Hercules, CA) set at 450 V, 500°F. After 48 hours in culture, 1.5 mg/ml geneticin was added to the medium and continuously maintained for 4 weeks. Stable transformants exhibiting GFP fluorescence were sorted by flow cytometry (FACStar,
10 Becton-Dickinson Immunocytometry Systems, San José, CA) using an FL1 sorting gate. The FL1-positive cells were dilutionally cloned in 96 well culture plates. pEGFP-PL scramblase transformants expressing the 62 kDa GFP-PL scramblase fusion protein were identified by Western
15 blotting with anti-GFP and with anti-PL-306-W318 antibodies. Western blotting of pEGFP-C2 transformants (without insert) confirmed presence of 27 kDa GFP. Clones expressing various amounts of GFP-PL scramblase were each expanded for functional assay, along with
20 comparable GFP-expressing clones serving as controls.

Fluorescence Microscopy. Cell clones transfected with pEGFP-C2-PL scramblase or pEGFP-C2 were deposited on glass microscopy slides using a Cytospin 3 (Shandon, Inc., Pittsburgh, PA). Phase contrast and fluorescence
25 microscopy was performed with a ZEISS AXIOSKOP microscope (Carl Zeiss, Inc., Thornwood, NY) equipped for epifluorescence, and images were recorded with a MC100 camera system. The exposure times for photography of

fluorescence was 80-200 seconds under automatic control using Kodak Ektachrome 1600 film.

Western Blot Analysis. Western blotting of GFP and PL scramblase antigens was performed using 1.5×10^6 cells per lane. After washing in HBSS, supernatants were removed, and the cell pellets extracted with 2% (v/v) NP-40 in 5 mM EDTA, 50 mM benzamidine, 50 mM N-ethyl maleimide, 1 mM phenylmethanesulfonyl fluoride, 1 mM leupeptin in HBSS. After removal of insoluble material (250,000 x g, 30 minutes, 4°C), the samples were denatured at 100°C in 10% (w/v) SDS sample buffer containing 2% β -mercaptoethanol. Following SDS-PAGE and transfer to nitrocellulose, the blocked membrane was incubated with either 10 μ g/ml of rabbit anti-PL scramblase-E306-W318, or 1/10,000 dilution of mouse anti-GFP. The blots were developed with the horseradish peroxidase conjugate of either goat anti-rabbit IgG or goat anti-mouse IgG, respectively, using SuperSignal ULTRA chemiluminescence.

Measurement of cell surface PS. Calcium ionophore-induced exposure of PS on the surface of all cell lines analyzed was detected by the specific binding of coagulation factor Va (light chain) as previously described (P.J. Sims, *et al.*, *J. Biol. Chem.* 263:18205-18212, 1988; H. Kojima, *et al.*, *J. Clin. Invest.* 94:2237-2243, 1994). Briefly, cells were washed twice to remove serum proteins and suspended (2×10^6 cells/ml) at 37°C in RPMI 1640 supplemented with 0.1% BSA, 20 mM HEPES, and

adjusted to 1.2 mM free $[Ca^{2+}]$. At time=0, A23187 (0 or 2 μ M final concentration) was added from 1 mM stock solution in DMSO, and at times indicated in figure legends, the reaction was stopped by addition of 6 mM EGTA. PS exposed on the cell surface at each time point was detected by incubating (10 minutes, room temperature) 50 μ l of the cell suspension with 10 μ g/ml FVa, followed by 10 μ g/ml anti-FVa, to detect the cell-bound FVa light chain. After staining with 10 μ g/ml Tri-Color conjugated goat anti-mouse IgG (CALTAG Laboratories, Burlingame, CA), single-cell fluorescence was quantitated by flow cytometry (FL3 channel, FACScan, Becton Dickinson Immunocytometry Systems). Use of Tri-Color conjugate to detect cell-bound FVa enabled simultaneous measurement of cell-associated GFP fluorescence in cell lines transformed with the pEGFP-C2 expression plasmid (fluorescence of GFP detected in FL1 channel). In experiments in which cell lysis was monitored by uptake of propidium iodide, cells were stained for bound FVa with FITC-conjugate of goat anti-mouse IgG (FL1 channel) substituting for Tri-Color conjugate, and propidium iodide was detected in FL3 channel. Propidium iodide (0.5 μ g/ml) was added immediately before dilution for flow cytometry.

Prothrombinase Assay. Prothrombinase activity of Raji cells was determined by modification of methods previously described for platelets, using the chromogenic thrombin substrate S2238 (P.J. Sims, et al., supra,

1988). Briefly, 1×10^5 Raji cells (transfected with either pEGFP-C2 or pEGFP-C2-PL scramblase) were suspended in 200 μ l HBSS containing 1% BSA in the presence of 2 nM FVa, 1.4 μ M prothrombin 2.5 mM CaCl_2 , and 4 μ M dansylarginine-N-(3-ethyl-1,5-pentanediy1)amide (to inhibit feed-back activation by thrombin), and incubated at 37°C. Ca^{2+} -ionophore A23187 (2 μ M), or DMSO (as solvent control) was added, and prothrombin conversion was initiated by addition of 2 nM Fxa. Thrombin generation was stopped after 2 minutes by dilution into 10 mM EGTA and samples were stored on ice. Aliquots were transferred to a 96-well plate, and thrombin generated was assayed in TBS containing 1% BSA in presence of 150 μ M S2238 by monitoring time-dependent changes in absorbance at 405 nm using a Thermo_{max} plate reader (Molecular Devices, Sunnyvale, CA). Thrombin activity was calculated using purified thrombin as standard.

C. Results

Analysis of PL scramblase in various human cell lines. Proteoliposomes reconstituted with erythrocyte PL scramblase exhibit accelerated transbilayer movement of fluorescent phospholipids in response to added Ca^{2+} , similar to the observed effect of calcium on the endofacial surface of the red cell membrane (Q. Zhou, et al., supra, 1997; J.G. Stout, et al., J. Clin. Invest. 99:2232-2238, 1997; Bassé, et al., J. Biol. Chem. 271:17205-17210, 1996). In order to determine whether this same protein is responsible for mediating the

accelerated egress of plasma membrane PS that is observed under conditions of elevated cytosolic $[Ca^{2+}]_c$, we undertook to determine whether the level of expression of PL scramblase in various human cell lines correlated to the induced movement of PS to the surface of these cells. When challenged with a calcium ionophore, human cell lines exhibit considerable differences in the extent to which PS is mobilized to the cell surface. Among the cells tested, Raji, HL60, and Dami were notably unresponsive to A23187, whereas HEL, W9 (an EBV-transformed normal B-lymphocyte), and Jurkat showed notably robust responses. This apparent cell type-specific variability in response to induced elevation of $[Ca^{2+}]_c$ was consistently maintained through many months of passage in culture, suggesting it reflected an inherent property of each cell line. As shown in Fig. 8, we also observed considerable differences in the content of PL scramblase protein among these various cell lines, and the sensitivity of these various cell lines to induced exposure of plasma membrane PS (lower panel) generally correlated with the amount of cellular PL scramblase protein detected by Western blotting (upper panel): Those cell lines that were most responsive to induced elevation of $[Ca^{2+}]_c$ (HEL, W9, Jurkat) also expressed greatest amounts of PL scramblase antigen, whereas cell lines with attenuated response to $[Ca^{2+}]_c$ (Raji, HL60, Dami) contained relatively little of this protein. Cell lines Molt-4 and K562 showed intermediate responses to

elevated $[Ca^{2+}]_c$ and expressed intermediate levels of PL scramblase antigen.

Fig. 8 depicts western blot analysis of PL scramblase in various human cell lines. Constitutive expression of PL scramblase was analyzed in the human cell lines indicated. Upper Panel: Results obtained by Western blotting with antibody specific for PL scramblase carboxyl terminal residues E306-W318 (see *Materials & Methods*). Each lane contains the total protein extract of 1.5×10^6 cells. Lower Panel: Cumulative results of three separate experiments performed as follows: The cells indicated were washed and suspended at $37^\circ C$ in the presence of 1.2 mM free Ca^{2+} , and 2 μM A23187 was added. At times shown (*abscissa*), EGTA was added and cells analyzed for surface exposed PS as detected by cell-bound FVa light chain (see *Materials and Methods*). Data plotted represent the mean increase (\pm SD) in number of cells that stained positive for surface PS after 5 minutes incubation with ionophore, after correction for initial background of PS-positive cells before addition of ionophore (time=0). Background number of cells that exposed PS in absence of ionophore was always <15% except in case of HEL, where this background ranged between 15-30%.

These relatively large differences in cell line-specific expression of this protein was also consistently observed despite repeated passage in culture, and was found to correspond to marked differences in level of

specific mRNA as detected by Northern blotting with PL
scramblase cDNA (Q. Zhou, et al., supra, 1997), and data
not shown). We also noted that those cell lines with the
highest content of PL scramblase generally exhibited a
5 higher background of PS exposed on the surface in absence
of added ionophore. This was most notable for HEL for
which approximately 15-30% of the cells were consistently
found to expose PS prior to addition of A23187 (see
Discussion).

10 Membrane changes underlying ionophore response. In
order to determine whether the increase in PS exposure in
ionophore-treated cells reflected facilitated movement of
PS from inner to outer leaflets of the plasma membrane,
or, greater sensitivity of the plasma membrane to lytic
15 disruption, FVa binding to the cell surface was monitored
simultaneously with uptake of propidium iodide, as a
measure of cell lysis. As illustrated for the human B-
lymphocyte lines W9 (high content of PL scramblase) and
Raji (low content of PL scramblase), the induced movement
20 of PS to the cell surface was found to precede uptake of
propidium iodide, suggesting that the elevation of $[Ca^{2+}]_i$
induces a collapse of transmembrane PL asymmetry before
onset of lysis. In the case of Raji cells which are
virtually devoid of PL scramblase (see Fig. 8), a general
25 insensitivity of the plasma membrane to either ionophore-
induced PS exposure or to lysis was also apparent.

Transfection of the Raji cell line with pEGFP-C2-PL
scramblase. In order to confirm that the extent to which

PS moves to the cell surface with elevation of $[Ca^{2+}]_i$ actually depends upon the plasma membrane content of PL scramblase, we stably transformed Raji, a cell line exhibiting low endogenous PL scramblase expression by transfection with plasmid pEGFP-C2-PL scramblase. This plasmid expresses PL scramblase as a fusion protein with green fluorescent protein (GFP), facilitating flow cytometric sorting of transformants for subsequent cloning and detection of the expressed recombinant protein in selected clones. The decision to attach GFP to the amino terminus of PL scramblase was based on prior evidence that the carboxyl terminus of the protein is membrane inserted and essential for function, and the observation that other amino terminal fusion constructs of PL scramblase expressed in *E. coli* retained the same activity of the unmodified PL scramblase polypeptide when reconstituted in proteoliposomes (Q. Zhou, *et al.*, *supra*, 1997), and unpublished data). The expression of the full-length GFP-PL scramblase fusion protein in selected transformed clones was confirmed by Western blotting with antibody specific for GFP, and with antibody raised against peptide sequence of the carboxyl terminus of human PL scramblase. As illustrated by fluorescence micrographs shown in Fig. 9, clones that expressed the GFP-PL scramblase fusion protein showed a distinct rim appearing pattern of fluorescence, consistent with trafficking of GFP-PL scramblase to the plasma membrane. Fig. 9 illustrates fluorescence micrographs of GFP-PL

scramblase transformed Raji cells. Fluorescence photomicrography of GFP fluorescence expressed in the transformed Raji clones was performed as described in *Materials and Methods*. Fig 9A shows fluorescence of cells expressing GFP; Fig. 9B shows cells transfected with pEGFP-C2-PL scramblase plasmid and expressing GFP-PL scramblase fusion protein. Data of single experiment, representative of results obtained for all clones transfected with either pEGFP-C2 or pEGFP-C2-PL scramblase. By contrast, clones that expressed GFP alone exhibited diffuse fluorescence throughout the cytoplasm, with no obvious staining of the plasma membrane. These data provide the first direct evidence that PL scramblase cDNA encodes a protein that predominantly trafficks to the plasma membrane under normal conditions of cell growth.

Analysis of PS mobilizing function in GFP-PL scramblase transformants. After geneticin selection, clonal populations of transformed Raji cells expressing comparable levels of either GFP-PL scramblase or GFP (transformed with pEGFP-C2 lacking insert) were analyzed for their capacity to mobilize PS to the cell surface. In response to an A23187-induced elevation of $[Ca^{2+}]_c$, transformants expressing the GFP-PL scramblase fusion construct showed a marked increase in both the rate and extent that PS became exposed on the cell surface, when compared to either the identically-treated parental Raji cell line or to GFP-expressing clones transformed with

pEGFP-C2 vector alone. As was also evident from these data, in the absence of ionophore, we consistently noted a small but reproducible increase in the background level of PS exposure in transformants expressing GFP-PL

5 scramblase protein, when compared to either the parental Raji cell lines or to GFP-expressing clones transformed with vector alone.

Induction of membrane procoagulant function through expression of PL scramblase. In order to confirm that

10 the increased expression of FVa binding sites detected upon activation of GFP-PL scramblase transformed clones reflected an increase in the procoagulant (clot-promoting) properties of the plasma membrane of these cells, the capacity of GFP-PL scramblase transformed

15 cells to provide catalytic membrane surface for the prothrombinase (FVaXa) enzyme complex was compared to clones expressing GFP alone. These data confirmed that expression of recombinant PL scramblase in the Raji cell line was also accompanied by an increase in cell capacity
20 to catalyze the prothrombinase reaction upon entry of calcium into the cytosol.

Level of expression of PL scramblase regulates capacity of to mobilize PS to the cell surface. In order to confirm the apparent correlation between endogenous
25 cell content of PL scramblase and plasma membrane sensitivity to elevated $[Ca^{2+}]_i$, that is evident when different human cell lines are compared (see Fig. 8), we analyzed multiple Raji clones that were stably

transfected with either GFP-PL scramblase or with GFP vector alone (Fig. 10). Fig. 10 illustrates that the level of expression of PL scramblase determines plasma membrane sensitivity to intracellular Ca^{2+} . The relationship between level of recombinant protein expressed (GFP fluorescence detected in FL1 channel; *abscissa*) and numbers of cells that expose PS after 2 minutes incubation with A23187 (*ordinate*) is plotted for multiple transformed Raji clones. Analysis was gated to include only those cells distinctly positive for GFP fluorescence (FL1 channel), and cell-bound FVa was stained with Tri-color conjugate and detected in FL3 channel (see *Materials and Methods*). Analysis was performed on all cells positive for GFP fluorescence.

Open symbols indicate individual clones stably transformed by transfection with pEGFP-C2; closed symbols indicate individual clones stably transformed with pEGFP-PL scramblase. Data of single experiment, representative of three so performed. These experiments confirm that the capacity of GFP-PL scramblase transformants to mobilize PS to the cell surface generally correlates with the amount of the expressed GFP-PL scramblase fusion protein, whereas this cell response to increased $[\text{Ca}^{2+}]_i$ is unaffected by cell content of GFP. In addition to confirming the role of PL scramblase in the plasma membrane response to $[\text{Ca}^{2+}]_i$, these data suggest that the capacity to mobilize PS to the cell surface and thereby support plasma clotting in activated, injured or

apoptotic cells exposed to elevated $[Ca^{2+}]_c$ can be altered by changing the level of expression of PL scramblase expressed in the plasma membrane.

Discussion

5 These results provide the first evidence that the PL
scramblase protein identified in the erythrocyte membrane
and implicated in $[Ca^{2+}]_c$ -induced remodeling of membrane
phospholipids actually functions to induce accelerated
transbilayer movement of plasma membrane phospholipid in
10 human cells that express this protein. Our results also
confirm that the level of expression of plasma membrane
PL scramblase can determine the extent to which PS is
mobilized to the cell surface upon elevation of $[Ca^{2+}]_c$,
and suggest that this protein normally functions to
15 mediate the redistribution of plasma membrane
phospholipids in response to the entry of calcium into
the cytosol. Furthermore, these data provide the first
indication that the movement of PS and other procoagulant
aminophospholipids from plasma membrane inner leaflet to
20 the cell surface can be manipulated by selectively
altering the level of expression of a particular cellular
protein, either through direct transfection with the PL
scramblase cDNA, or potentially, by another intervention
affecting cellular expression of functional PL scramblase
25 protein.

Whereas these experiments suggest that direct
activation of plasma membrane PL scramblase is
responsible for the increased cell surface exposure of PS

that is observed in various activated, injured or apoptotic cells exposed to elevated $[Ca^{2+}]_c$, we cannot exclude the possibility that there are other cellular components that contribute to the accelerated movement of PS from inner to outer plasma membrane leaflet under these conditions. In particular, whereas PL scramblase has been shown to mediate the bidirectional movement of PS and other phospholipids between membrane leaflets, it has been suggested that there is also a PS-selective pathway in the platelet plasma membrane, designated "PS floppase", which mediates vectorial movement of PS from inner to outer plasma membrane leaflet (P. Gaffet, et al., Biochemistry 34:6762-6769, 1995). Experimental evidence for the existence of this vectorial and headgroup-selective PS floppase pathway in platelet or other cell membranes remains controversial (R.F.A. Zwaal, et al., supra, 1997; P. Williamson, et al., Biochemistry 31:6355-6360, 1995; C.-P. Chang, et al., J. Biol. Chem. 268:7171-7178, 1993), and awaits identity of a $[Ca^{2+}]_c$ -activated and PS-selective transporter that is distinct from the plasma membrane PL scramblase found in platelets and erythrocytes, a protein that does not exhibit apparent selectivity for the PS headgroup (J.G. Stout, et al., supra, 1997; P. Comfurius, et al., Biochemistry 35,7631-7634, 1996; F. Bassé, et al., supra, 1996).

In addition to conferring increased sensitivity of the plasma membrane to ionophore-induced elevation of $[Ca^{2+}]_c$, we generally observed a higher background of PS

exposure (in absence of ionophore) in those transfected cell clones expressing large amounts of the GFP-PL scramblase fusion protein. This elevated background PS exposure was also observed in the case of untreated HEL, the cell line containing the highest endogenous content of PL scramblase. Although we suspect that this increased background reflects the enhanced sensitivity of the plasma membrane of these cells to any adventitial elevation of $[Ca^{2+}]_e$ during cell processing for assay, we cannot exclude the possibility that these cells are also inherently more fragile due to the large amounts of PL scramblase that is inserted into the plasma membrane.

While the movement of plasma membrane PS to the cell surface at elevated $[Ca^{2+}]_e$ can be demonstrated in a variety of cells and tissues (R.F.A. Zwaal, et al., supra, 1997; P. Devaux, supra, 1991), we detect marked differences in the levels of PL scramblase mRNA and protein among different human cell types, which is generally reflected by corresponding differences in sensitivity to this $[Ca^{2+}]_e$ -induced collapse of plasma membrane PL asymmetry (see Fig. 8, and Q. Zhou, et al., supra, 1997). Although the transcriptional regulation of the PL scramblase gene remains to be determined, it is of interest to note that such cell or tissue-specific differences in PL scramblase expression has the potential to significantly affect the biological properties of the cell. In particular, we note that the content of PL scramblase in human platelet is approximately 10-fold

greater than that of the erythrocyte, which is consistent with the respective PS-mobilizing potential and different roles of these two cells in contributing procoagulant membrane surface for thrombin generation during blood clotting (Q. Zhou, *et al.*, *supra*, 1997). In addition to the relatively high levels of PL scramblase identified in circulating human platelets, this protein was also most abundant in the cell line HEL, whereas only small amounts of this protein (and low PL scramblase activity) was detected for Dami (Fig. 8), two human cancer cell lines exhibiting partial megakaryocytic-like properties. It is also noteworthy that several of the lymphoma-derived cell lines (e.g., Raji, MOLT-4) express considerably reduced levels of PL scramblase, and also show distinctly attenuated PS exposure in response to elevated $[Ca^{2+}]_i$, when compared to either peripheral blood leukocytes or to EBV-transforms of normal lymphocytes (Fig. 8). The collapse of plasma membrane phospholipid asymmetry is a relatively early event in apoptosis of lymphocytes and other cells, and the consequent exposure of PS on the cell surface is thought to contribute to phagocytic removal of such cells by scavenger macrophages (V.A. Fadok, *et al.*, *J. Immunol.* 148:2207-2216, 1992; B. Verhoven, *et al.*, *J. Exp. Med.* 182:1597-1601, 1995). It is therefore of interest to consider whether the apparent resistance of certain lymphoma-derived cell lines to such $[Ca^{2+}]_i$ -induced remodeling of plasma membrane phospholipids might contribute to the proliferative

potential and in vivo survival of these or other transformed cells.

EXAMPLE 4: Induction of Programmed Cell Death is Related to the Cellular Content of PL Scramblase

5 A. Summary

Human cell lines expressing various levels of PL
scramblase protein were exposed to agents or conditions
of cell culture known to promote apoptosis (programmed
cell death). Analysis of these cell lines revealed that
10 the number of cells induced to undergo apoptosis
generally correlated with the level of expression of PL
scramblase protein, with those cell lines expressing high
levels of PL scramblase being most sensitive to induction
of apoptosis while cell lines expressing low levels of PL
15 scramblase being most resistant to induction of
apoptosis. The human Burkitts B-lymphoma cell line Raji
was found to express low levels of PL scramblase and to
be markedly resistant to induction of apoptosis.
Transfection of Raji cells with plasmid containing cDNA
20 for PL scramblase resulted in increased sensitivity of
this cell to the induction of apoptosis, demonstrating
that expression of PL scramblase in a normally apoptosis-
resistant cancer cell line promotes programmed death of
the cancer cell.

25 B. Materials and Methods

Materials. All restriction enzymes were from New
England BioLabs, Inc. (Beverly, MA). KlenTaq polymerase
and pEGFP-C2 vector were from CLONTECH Laboratories (Palo
Alto, CA). Bovine coagulation factor Va (Fva). OPTI-MEM

and geneticin were from Life Technologies (Gaithersburg, MD). Fetal bovine serum, RPMI 1640, Cell Dissociation Solution, Hank's Balanced Salt Solution (HBSS), Protein A Sepharose-CL4B, leupeptin, and BSA were from Sigma Chemical Co. (St. Louis, MO). UltraLink Iodoacetyl resin and SuperSignal ULTRA Chemiluminescence Kit were from Pierce Chemical Co. (Rockford, IL). All other chemicals were of reagent grade.

Antibodies: *Anti-GFP:* murine monoclonal antibody against green fluorescent protein (GFP) was from CLONTECH Laboratories. *Anti-FVa:* murine monoclonal antibody V237 reactive against human or bovine factor Va light chain was the generous gift of Dr. Charles T. Esmon (Oklahoma Medical Research Fndn, Oklahoma City, OK). *Anti-PL Scramblase-E306-W318:* Rabbit antibody raised against the carboxyl terminal peptide sequence E306-W318 of human PL scramblase has previously been described (Q. Zhou, et al., supra, 1997). The IgG fraction was isolated on protein A-Sepharose-CL4B and the peptide-reactive antibody purified by affinity chromatography on peptide [Cys]-ESTGSQEQKSGVW (SEQ ID NO:5) coupled to UltraLink Iodoacetyl resin. The IgG fraction of anti-human FAS ligand was from Clontech (Sunnyvale, CA).

Cell culture: Human cancer cell lines erythroleukemic HEL, promyelocytic leukemia HL-60, chronic myelogenous leukemia K562, lymphoblastic leukemia MOLT-4, acute T-cell leukemia Jurkat, Burkitt's lymphoma Raji, and megakaryocytic DAMI were from American Type

Culture Collection (Rockville, MD) and cultured in RPMI 1640 containing 10% fetal bovine serum. EBV-transformed cell line W9 established from peripheral B-lymphocytes of a normal donor was maintained as previously described (H.

5 Kojima, et al., 1994).

Plasmid Construction: Human PL scramblase cDNA insert was released from plasmid pMAL-C2-PL scramblase (Q. Zhou, et al., supra, 1997) by double cutting with *EcoRI* and *SalI*, respectively, and then ligated into
10 pEGFP-C2 vector using the same restriction site. The pEGFP-C2-PL scramblase plasmid was amplified from single clones in *E. coli* strain Top 10, and the orientation and reading frame of the insert confirmed by sequencing on an ABI DNA Sequencer Model 373 Stretch (Perkin Elmer-
15 Applied Biosystems, Foster City, CA) using PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit.

Transfection of Raji cells with pEGFP-PL scramblase.

1.6 x 10⁷ Raji cells were electroporated with 160 µg plasmid DNA (pEGFP-C2-PL scramblase or pEGFP-C2) in a
20 total volume of 0.8 ml OPTI-MEM, using Gene Pulse Electroporator (Bio-Rad Laboratories, Hercules, CA) set at 450 V, 500°F. After 48 hours in culture, 1.5 mg/ml geneticin was added to the medium and continuously maintained for 4 weeks. Stable transformants exhibiting
25 GFP fluorescence were sorted by flow cytometry (FACStar, Becton-Dickinson Immunocytometry Systems, San José, CA) using an FL1 sorting gate. The FL1-positive cells were dilutionally cloned in 96 well culture plates. pEGFP-PL

scramblase transformants expressing the 62 kDa GFP-PL
scramblase fusion protein were identified by Western
blotting with anti-GFP and with anti-PL-306-W318
antibodies. Western blotting of pEGFP-C2 transformants
5 (without insert) confirmed presence of 27 kDa GFP.
Clones expressing various amounts of GFP-PL scramblase
were each expanded for functional assay, along with
comparable GFP-expressing clones serving as controls.

Fluorescence Microscopy. Cell clones transfected
10 with pEGFP-C2-PL scramblase or pEGFP-C2 were deposited on
glass microscopy slides using a Cytospin 3 (Shandon,
Inc., Pittsburgh, PA). Phase contrast and fluorescence
microscopy was performed with a ZEISS AXIOSKOP microscope
(Carl Zeiss, Inc., Thornwood, NY) equipped for
15 epifluorescence, and images were recorded with a MC100
camera system. The exposure times for photography of
fluorescence was 80-200 seconds under automatic control
using Kodak Ektachrome 1600 film.

Western Blot Analysis. Western blotting of GFP and
20 PL scramblase antigens was performed using 1.5×10^6 cells
per lane. After washing in HBSS, supernatants were
removed, and the cell pellets extracted with 2% (v/v) NP-
40 in 5 mM EDTA, 50 mM benzamidine, 50 mM N-ethyl
maleimide, 1 mM phenylmethylsulfonyl fluoride, 1 mM
25 leupeptin in HBSS. After removal of insoluble material
(250,000 x g, 30 minutes, 4°C), the samples were
denatured at 100°C in 10% (w/v) SDS sample buffer
containing 2% β -mercaptoethanol. Following SDS-PAGE and

transfer to nitrocellulose, the blocked membrane was incubated with either 10 μ g/ml of rabbit anti-PL scramblase-E306-W318, or 1/10,000 dilution of mouse anti-GFP. The blots were developed with the horseradish
5 peroxidase conjugate of either goat anti-rabbit IgG or goat anti-mouse IgG, respectively, using SuperSignal ULTRA chemiluminescence.

Induction of apoptosis: Apoptosis was induced either by (1) addition of 60 μ M etoposide to the cell
10 culture (2) by removal of fetal bovine serum from the culture medium (serum starvation). Cells were maintained in sterile culture flasks in 37°C incubator under each of these conditions for 72 hours, during which cell aliquots were obtained for assay (times indicated in figure
15 legends).

Assays for Apoptosis: The induction of apoptosis was detected by (1) egress of PS to the cell surface (2) activation of cellular caspase enzymes (3) cleavage of nuclear DNA. Egress of PS to the cell surface was
20 measured by the binding of Factor Va (light chain) or by the binding of FITC-annexin V, detected by flow cytometry. Cleavage of nuclear DNA was measured either by a decrease in propidium iodide staining of the nucleus, according to published methods (ref), or by the
25 measured incorporation of FITC-dUTP into 5'-ends of chromosomal DNA, catalyzed by the enzyme terminal deoxyribose transferase (TUNEL Assay Kit, Clontech) according to the manufacturer's protocol. In each assay,

fluorescence was detected by flow cytometry (FACScan, Becton Dickinson), using the FL1 channel to detect FITC or the FL3 channel to detect propidium iodide staining of the nucleus. Forward and side scatter gates were set to
5 exclude cell debris.

Measurement of cell surface PS. Exposure of PS on the surface of all cell lines analyzed was detected by the specific binding of coagulation factor Va (light chain) as previously described (P.J. Sims, et al., J. Biol. Chem. 263:18205-18212, 1988; H. Kojima, et al., J. Clin. Invest. 94:2237-2243, 1994). Briefly, cells were
10 washed twice to remove serum proteins and suspended (2×10^6 cells/ml) at 37°C in RPMI 1640 supplemented with 0.1% BSA, 20 mM HEPES, and adjusted to 1.2 mM free $[Ca^{2+}]$. At
15 time=0, A23187 (0 or 2 μ M final concentration) was added from 1 mM stock solution in DMSO, and at times indicated in figure legends, the reaction was stopped by addition of 6 mM EGTA. PS exposed on the cell surface at each
20 time point was detected by incubating (10 minutes, room temperature) 50 μ l of the cell suspension with 10 μ g/ml FVa, followed by 10 μ g/ml anti-FVa, to detect the cell-bound FVa light chain. After staining with 10 μ g/ml Tri-Color conjugated goat anti-mouse IgG (CALTAG
Laboratories, Burlingame, CA), single-cell fluorescence
25 was quantitated by flow cytometry (FL3 channel, FACScan, Becton Dickinson Immunocytometry Systems). Use of Tri-Color conjugate to detect cell-bound FVa enabled simultaneous measurement of cell-associated GFP

fluorescence in cell lines transformed with the pEGFP-C2 expression plasmid (fluorescence of GFP detected in FL1 channel). In experiments in which cell lysis was monitored by uptake of propidium iodide, cells were stained for bound FVa with FITC-conjugate of goat anti-mouse IgG (FL1 channel) substituting for Tri-Color conjugate, and propidium iodide was detected in FL3 channel. Propidium iodide (0.5 μ g/ml) was added immediately before dilution for flow cytometry.

10 C. Results

Role of PL scramblase in PS mobilization under conditions of induced apoptosis. As it appeared that the cellular content of PL scramblase determines the sensitivity of the plasma membrane to accelerated transbilayer migration of PS induced through elevation in cytoplasmic Ca^{2+} (see Example 3, supra), we considered whether the expression of this protein might also determine the extent to which PS is mobilized to cell surfaces under conditions of programmed cell death. As previously reported, the extent to which PS is exposed at cell surfaces when cells are subjected to conditions that ultimately result in their apoptotic death, vary widely between different cell lines, suggesting that the reorganization of plasma membrane phospholipids during programmed cell death is a regulated response inherent to particular cell lines (T. Frey, et al., J. Cytometry 28:253-263, 1997). In particular, both Raji and HL60 have recently been noted to be relatively resistant to

apoptotic induction of cell surface PS (D.L. Bratton, et al., supra, 1997; T. Frey, supra, 1997), consistent with the resistance we observe in the response of these cell lines to a direct elevation of $[Ca^{2+}]_c$ (See Example 3, above). We therefore compared PS exposure in the B-lymphoblast cell lines Raji (low level PL scramblase expression) and W9 (high level PL scramblase expression) under conditions of serum starvation which has been shown to induce apoptotic death in human B-lymphoblasts (T. Frey, supra, 1997).

Fig. 14 illustrates the role of PL scramblase in PS exposure during apoptosis of B-lymphoblasts. Referring to Fig. 14, PS exposure (measured with Fva light chain) in human B-lymphoblast cell lines during apoptosis induced by one day incubation in reduced serum (1% fetal bovine serum; *solid bars*). Open bars represent identically matched controls maintained continuously in 10% serum. Panel A compares the Raji (low endogenous PL scramblase expression) and W9 (high PL scramblase expression) cell lines. Panel B compares individual Raji cell clones that were stably transfected with either pEGFP-C2 (GFP) or with pEGFP-PL scramblase (GFP-PLS) and then induced to apoptosis under the conditions described for Panel A. Error bars denote mean \pm SD ($s=4$). Significance of the increase in PS exposure due to serum starvation by paired t-test, $*p<0.001$ (W9); $**<0.02$ (GFP-PLS).

As illustrated by Fig. 14 (panel A), when each cell line was incubated under conditions inducing apoptosis, PS exposure observed for W9 was always substantially elevated above that observed for Raji, consistent with both the greater quantity of PL scramblase protein expressed in the W9 cell line and this cell's increased sensitivity to PS mobilization through Ca^{2+} ionophore. This increased activity of the PL scramblase pathway in W9 versus Raji was also apparent when apoptosis was induced in these cells by exposure to etoposide (data not shown). Furthermore, we observed that the extent to which PS was mobilized to the surface of Raji cells undergoing apoptosis was increased through prior transfection of these cells to express the GFP-PL scramblase fusion protein (Fig. 14, panel B). These data suggest that the extent to which plasma membrane PS becomes exposed at the cell surface during induced apoptosis of lymphoblasts and other cell lines is directly related to the cellular content of PL scramblase protein.

Expression of PL scramblase promotes intracellular events that result in apoptotic cell death. In order to determine whether the level of expression of PL scramblase alters the capacity of a cell to respond to an external signal to undergo programmed cell death, we used the GFP-PL scramblase-transfected Raji cell lines to monitor fragmentation of nuclear DNA in response to known apoptotic stimuli. In these experiments, the transfected

cells were induced to apoptosis by either culture under serum starvation conditions or by challenge with etoposide. Nuclear degradation was monitored by TUNEL assay (Clontech) and data analyzed as per cent of all
5 cells induced to DNA fragmentation.

Fig. 15 illustrates transfection with cDNA encoding PL scramblase increases apoptotic response of human B-lymphoma line Raji. Raji cell lines were stably transfected with plasmid to express either GFP-PL
10 scramblase fusion protein (GFP-PLS; hatched bars) or pGFP vector as control (GFP; solid bars) and analyzed under conditions of induced apoptosis in cell culture. Also shown are data for non-transfected parental cell line Raji (Raji; open bars). Each cell line was incubated
15 overnight under either serum-starved conditions (0% serum) or in presence of 60 μ M etoposide (Etoposide). Control refers to identical cells maintained under normal culture conditions of 10% fetal bovine serum. Apoptosis was assessed by degradation of nuclear DNA, as measured
20 by TUNEL assay (Clontech) and is expressed as percent of total cells induced to undergo nuclear fragmentation. Data of a single experiment, representative of at least five similar experiments so performed.

As shown in Fig. 15, these experiments revealed that
25 whereas untransfected Raji cells or GFP-vector transfected Raji cell lines were highly resistant to induction of apoptosis, the Raji cell lines expressing the GFP-PL scramblase fusion protein were readily induced

to apoptosis, culminating in fragmentation of nuclear DNA. Consistent with these data, we also observed that the cell lines W9 and Jurkat (expressing high levels of endogenous PL scramblase) were markedly more sensitive to
5 induction of apoptosis than the cell line Raji (which normal expresses little PL scramblase (data not shown)). In addition to suggesting that the sensitivity of a cell to induction of programmed cell death can be related to the level of expression of PL scramblase, these
10 experiments establish that a transformed tumor cell line (the human Burkitts lymphoma cell line, Raji) that is normally resistant to apoptosis, can be altered through transfection with cDNA encoding PL scramblase to be sensitive to induction of programmed cell death.

SEQUENCE LISTING

<110> Sims, Peter J.
Zhao, Ji
Wiedmer, Therese

<120> METHODS AND COMPOSITIONS TO ALTER TISSUE SUSCEPTIBILITY
TO IMMUNE INJURY, TO PROGRAMMED CELL DEATH, AND TO
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<210> 7

<211> 43

<212> DNA

<213> Unknown

<400> 7

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<210> 8

<211> 38

<212> DNA

<213> Unknown

<400> 8

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<210> 9

<211> 43

<212> DNA

<213> Unknown

<400> 9

gcttgccctgc aggtcgacct acacacagcc ttcaaaaaaac atg 43

CLAIMS

We claim:

1. A method for extending the viability of mammalian cells or tissues comprising the step of inhibiting the expression of native PL scramblase within the cell or tissue.

2. The method of claim 1 wherein the inhibition is *in vitro*.

3. The method of claim 1 wherein the inhibition is *in vivo*.

4. The method of claim 1 wherein the mammalian cell or tissue is selected from the group consisting of a blood cell stored for transfusion, a hematopoietic stem cell, an endothelial cell, a pancreatic islet cell, a blood vessel, a skin or other tissue graft, or solid organ for transplantation.

5. The method of claim 1 wherein the inhibition is via a PL scramblase antisense RNA molecule.

6. The method of claim 1 wherein the cell or tissue is a human cell or tissue.

7. The method of claim 1 wherein expression is inhibited via a mutant or truncated form of PL scramblase protein that is inhibitory to endogenous PL scramblase protein.

8. The method of claim 7 wherein the truncated form of the protein is the translation product of an alternatively spliced PL scramblase mRNA.

9. The method of claim 7 wherein the mutant PL scramblase contains one or more non-conservative substitution of residues in the Asp²⁷³-to-Asp²⁸⁴ region of PL scramblase polypeptide.

10. The method of claim 1 wherein the function of cell PL Scramblase is inhibited by preventing fatty acylation of the nascent PL scramblase polypeptide.

11. The method of claim 1 wherein the function of cell PL scramblase is inhibited by deacylation of the mature PL scramblase protein.

12. a method of decreasing the viability, metastatic or invasive potential of cancer cells, cancerous tissue, or viral-infected cell by causing increased expression or activity of PL scramblase protein within the cell or tissue.

13. The method of claim 12 wherein the cancer cell represents a leukemia, lymphoma, carcinoma, adenoma, sarcoma, or other transformed cell type with oncogenic, metastatic, or tumorigenic potential.

14. The method of claim 12 wherein the cell is infected with a human retrovirus, human leukemia virus, human adenovirus.

15. The method of claim 12 wherein the cancer cell, cancerous tissue or viral-infected cell is transfected to express an exogenous PL scramblase coding sequence.

16. The method of claim 12 wherein the cancer cell, cancerous tissue or viral-infected cell expresses exogenous PL scramblase protein.

17. The method of claim 12 wherein the cancer cell, cancerous tissue or viral-infected cell is within a human patient.

18. The method of claim 12 wherein the increased expression of PL scramblase protein is achieved by decreasing expression of alternatively spliced transcripts of PL scramblase within the cancer cell,
5 cancerous tissue or viral-infected cell.

19. A method of determining the status of a patient's cancer comprising the steps of analyzing the level of PL scramblase RNA or PL scramblase protein within cancer cells and correlating the level with a standard curve.

20. A method of diagnosing metastatic and invasive potential or growth potential of a cancer cell or cancerous tissue comprising the step of analyzing the amount of PL scramblase RNA or protein within a patient's cell sample.

21. The methods of claims 19 or 20 wherein the level of PL scramblase RNA is measured by *in situ* hybridization or Northern blotting with PL scramblase cDNA or by the polymerase chain reaction using oligonucleotide primers based on sequence of human PL scramblase cDNA.

22. The method of claims 19 or 20 wherein the level of PL scramblase protein is measured by immunofluorescence, flow cytometry, Western blotting, radioimmunoassay, or by ELISA assay using antibody that specifically binds PL scramblase protein.

23. A method for inducing apoptosis in a cell comprising the step of increasing expression of PL scramblase within the cell.

24. The method of claim 23 wherein the increase in PL scramblase expression is by activating the promoter of an endogenous PL scramblase gene.

25. The method of claim 23 wherein the increase in PL scramblase expression is by transfection of the cell with cDNA encoding PL scramblase.

26. The method of claim 23 wherein the cell is part of a tissue.

27. A method of diagnosing metastatic and invasive potential or growth potential of a cancer cell or cancerous tissue comprising the step of analyzing the sequence of a patient's PL scramblase gene, RNA or
5 protein for the presence or absence of mutations.

28. The method of claim 27 wherein the analysis comprises techniques selected from the group consisting of RFLP, sequencing by RT-PCR, Northern blotting, Western blotting, electrophoretic gels, protease digestion and
5 other techniques designed to analyze DNA, RNA or protein sequence.

1 cgccggccgcgtcgaccgaaaccaggagccgcgggtgttggcgcaaagggttactcccagac
 61 ccttttccggctgacttctgagaagggttgcgcagcagctgtgcccgacagtctagaggcg
 121 cagaagagggaagccatcgccctggcccggtctcttggaacctgtctcgctcgggagcgga
 181 aacagcgccagccagagaaactgttttaatcatggacaaacaaaactcacagatgaatgct
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 241 tctcaccggaaacaaaacttggcagttgggtatcctcctcagtatccaccgacagcattc
 11 S H P E T N L P V G Y P P Q Y P P T A F
 301 caaggacctccaggatatagtggctaccctgggccccagggtcagctacccacccccacca
 31 Q G P P G Y S G Y P G P Q V S Y P P P P
 361 gccggccattcaggtccttggcccgagctggctttcctgtcccaaatcagccagtgtataat
 51 A G H S G P G P A G F P V P N Q P V Y N
 421 cagccagtataataatcagccagttggagctgcaggggtaccatggatgccagcgccacag
 71 Q P V Y N Q P V G A A G V P W M P A P Q
 481 cctccattaaactgtccacctggattagaatatattaagtcagatagatcagatactgatt
 91 P P L N C P P G L E Y L S Q I D O I L I
 541 catcagcaaatgaacttctggaagttttaacagggttttgaaactaataacaaatgatga
 111 H O O I E L L E V L T G F E T N N K Y E
 601 attagaacagcttttggacagagggtttacttttgcagcggaagatactgattgctgtacc
 131 I K N S F G Q R V Y F A A E D T D C C T
 661 cgaaattgctgtgggccatctagaccttttaccttgaggattattgataatatgggtcaa
 151 R N C C G P S R P F T* L R I I D N M G Q
 721 gaagtcataactctggagagaccactaagatgtagcagctgttgttgccttgccttgcctt
 171 E V I T L E R P L R C S S C C C P C C L
 781 caggagatagaaatccaagctcctcctgggtgtaccaatagggttatgttattcagacttgg
 191 Q E I E I Q A P P G V P I G Y V I Q T W
 841 caccatgtctaccaaagttttacaattcaaaatgagaaaaagagaggatgtactaaaaata
 211 H P C L P K F T I Q N E K R E D V L K I
 901 agtggctccatgtgttgtgtgcagctgttgtggagatgttgattttgagattaaatctctt
 231 S G P C V V C S C C G D V D F E I K S L
 961 gatgaacagtggtgttgggttggcaaaattttccaagcactggactggaattttgagagaggca
 251 D E Q C V V G K I S K H W T G I L R E A
 1021 ttacagacgctgataaactttggaatccagttcccttttagaccttgatgttaaaatgaaa
 271 F T D A D N F G I Q F P L D L D V K M K
 1081 gctgtaatgattgggtgcctgtttcctcattgaacttcattgttttttgaaagcactggcagc
 291 A V M I G A C F L I D F M F F E S T G S
 1141 caggaacaaaaatcaggagtggttagtggttagtgaaagtctcctcaggaaatctgaa
 311 Q E Q K S G V W -
 1201 gtctgtatattgattgagactatctaaactcatacctgtatgaattaagctgtaaggcct
 1261 gtacgtctgggtgtataacttttgcttttcaaaattatagtttatcttctgtataactgatt
 1321 tataaagggtttttgtacattttttaataactcattgtcaatttgagaaaaaggacatatga
 1381 gtttttgcattttattaatgaaaacttcttttgaaaaactgctttaaaaaaaagtcgacgcg
 1441 gccgc

FIG. 1A

1 tctaaagactcaggaacacaaacctaattgcctcnaagttcagggtgctttttctccctg
 61 acttttagtctagtggagtagtgcagcacctatgcctttctgagaggagtctggagagctg
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 181 aaggcttgcaaatggaggctcctcgctcaggaacatacttgccagctgggtatgccccctc
 1 M E A P R S G T Y L P A G Y A P Q
 241 agtatcctccagcagcagtcacaggacctccagagcatactggacgccccacattccaga
 18 Y P P A A V Q G P P E H T G R P T F Q T
 301 ctaactaccaagtccccagctctgggttatccaggacctcaggctagctacacagttctcaa
 38 N Y Q V P Q S G Y P G P Q A S Y T V S T
 361 catctggacatgaagggttatgctgctacacggcttccctattcaaaataatcagactatag
 58 S G H E G Y A A T R L P I Q N N Q T I V
 421 tccttgcaaacactcagtggtgcccagcaccaccacctattctgaactgccccacctgggc
 78 L A N T Q W M P A P P P I L N C P P G L
 481 tagaatacttaaatcagatagatcagcttctgattcatcagcaagttgaacttctagaag
 98 E Y L N Q I D Q L L I H Q Q V E L L E V
 541 tcttaacaggctttgaaacaaataacaaatttgaaatcaagaacagcctcgggcagatgg
 118 L T G F E T N N K F E I K N S L G Q M V
 601 tttatgttgagtggaagatactgactgctgtactcgaatttgctgtgaagcgtctagac
 138 Y V A V E D T D C C T R N C C E A S R P
 661 ctttcaccttaagaatcctggatcatctgggccaagaagtcagactctggagcgacctc
 158 F T L R I L D H L G Q E V M T L E R P L
 721 tgagatgcagtagctgctgcttccccctgctgectccaggagatagaaatccagggtcctc
 178 R C S S C C F P C C L Q E I E I Q A P P
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 198 G V P I G Y V T Q T W H P C L P K L T L
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 218 Q N D K R E N V L K V V G P C V A C T C
 901 gctgttcagatattgacttttgagatcaagttctcttgatgaagtgactagaattggtaaga
 238 C S D I D F E I K S L D E V T R I G K I
 961 tcaccaagcagtggttctggttgtgtgaaagaggccttcacggattcggataactttggga
 258 T K Q W S G C V K E A F T D S D N F G I
 1021 tccaattccccgctagacctggagggtgaagatgaaagctgtgacgcttgggtgcttgcttcc
 278 Q F P L D L E V K M K A V T L G A C F L
 1081 tcatagattacatgttttttgagggtgtgagtaggaacagaaatccgacctgcagtagg
 298 I D Y M P F E G C E -
 1141 aatcaatgaaagaggacagagaagatctgaagtctacacnaaggagatcatatgattgaga
 1201 gacctggggcttttttgatttcttccattgaaatttctcagaatcaagctgttatacatgaa
 1261 gcatagtatgtaacatttttggttttcaaatggtagtttatcttttacattatttggaaatag
 1321 acctggataaattatctttatacacttctaaaaatatgcaccaaattcaagttaaaaaa
 1381 aaagacgaagagaagtgtatgttttaaaataaaacattttatggaaaagtaagttaaatc
 1441 ataactctgggattttatctttcatcttttgttcaatttaaaccttgttagtgctgatttta
 1501 ttataaaattgtactttactatcaaaccttagtttagttttatctttacagaaatcctccta
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 1621 ta

FIG. 1B

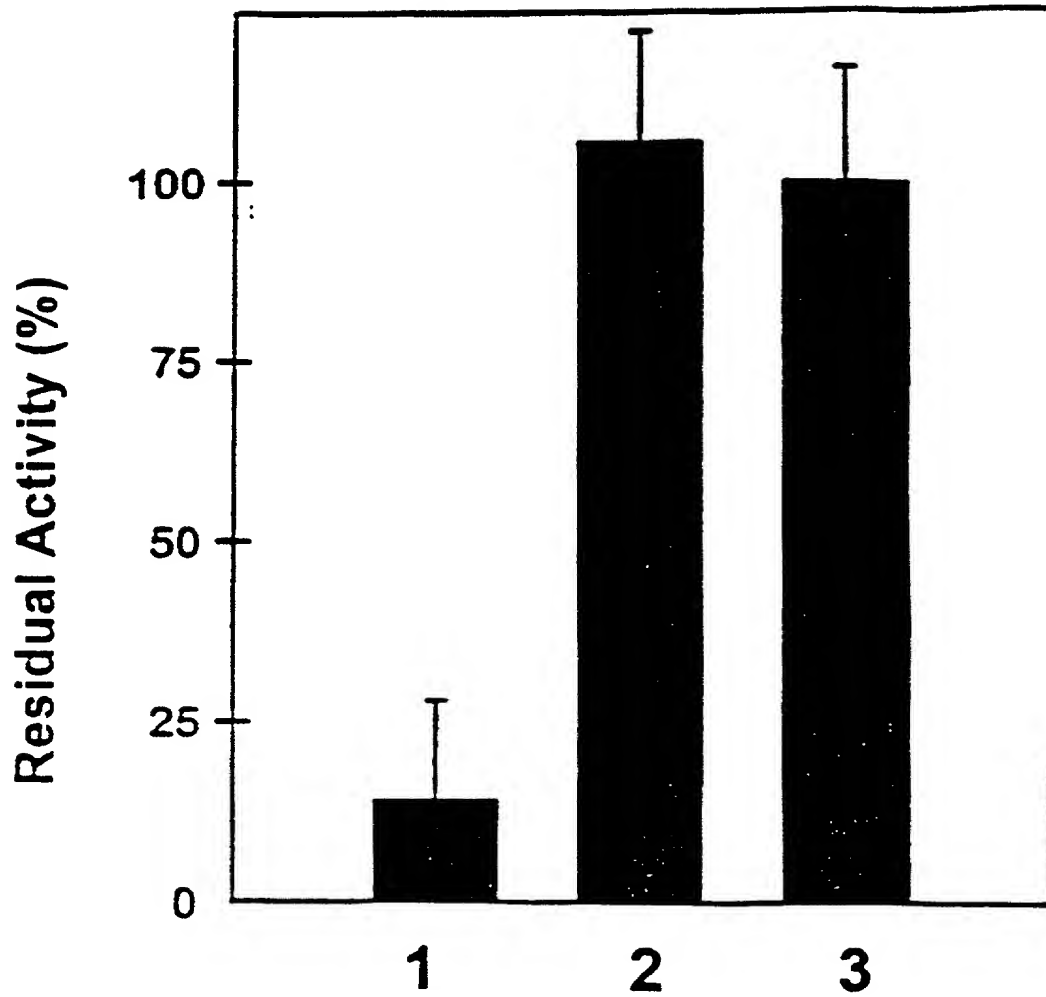


FIG. 2

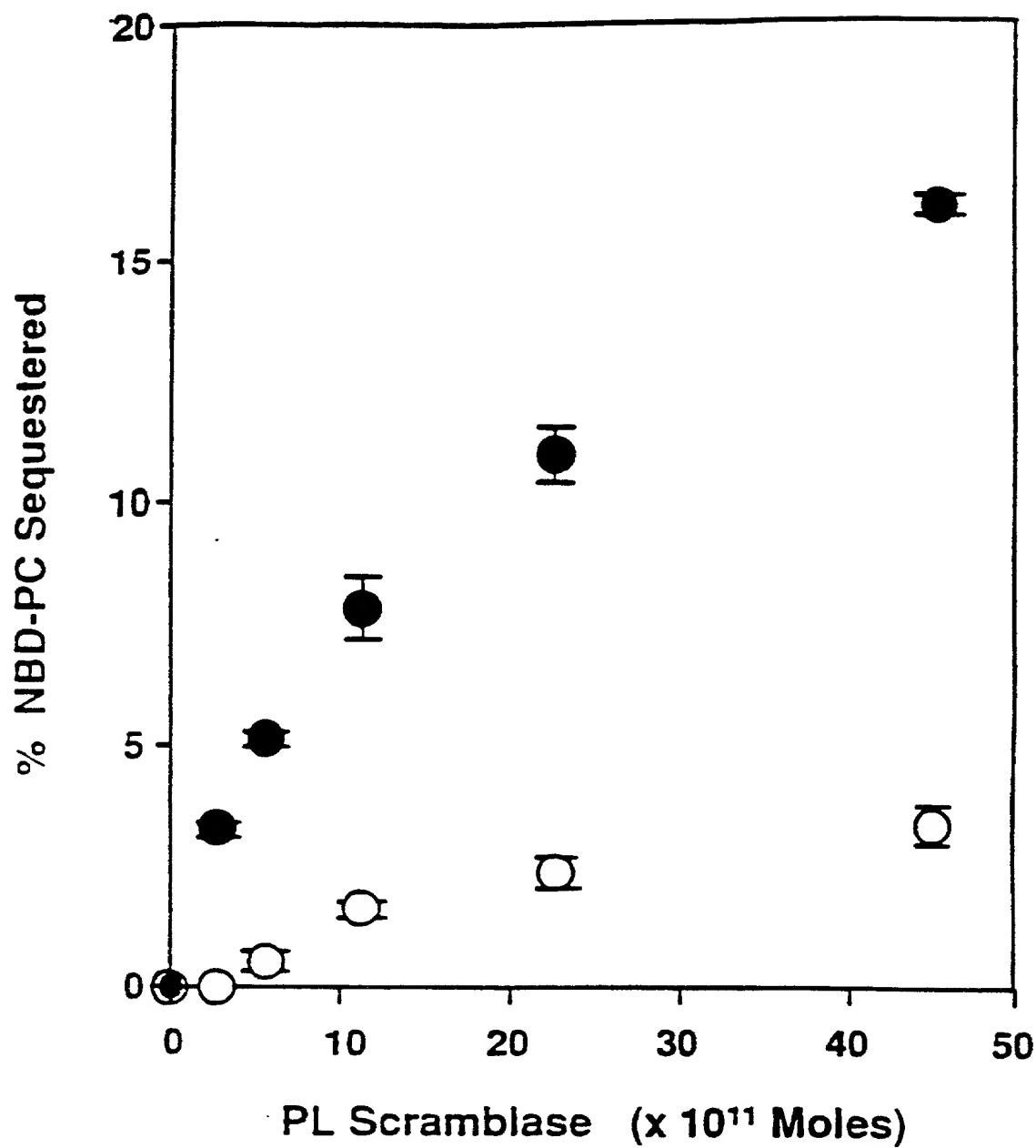


FIG. 3

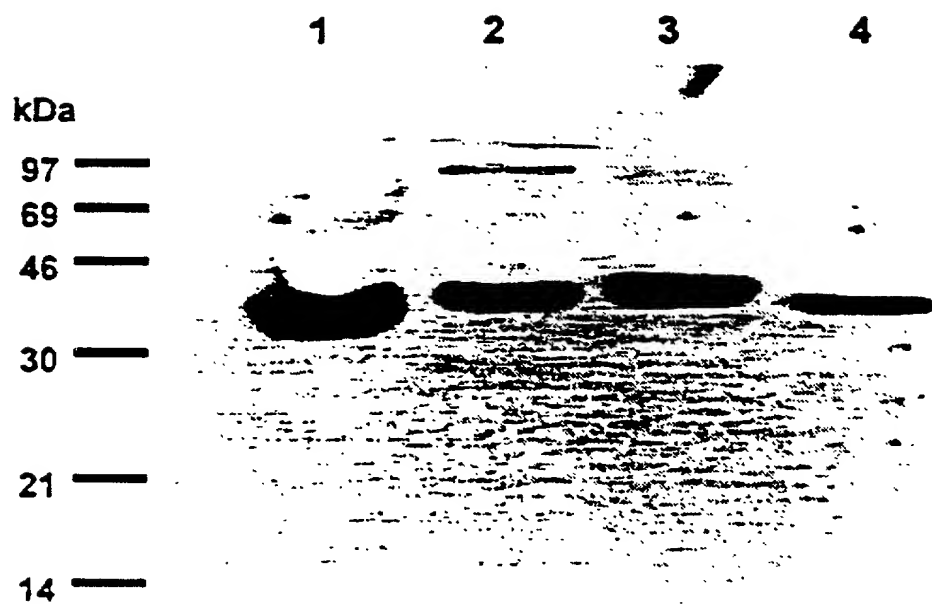


FIG. 4

FIG. 5

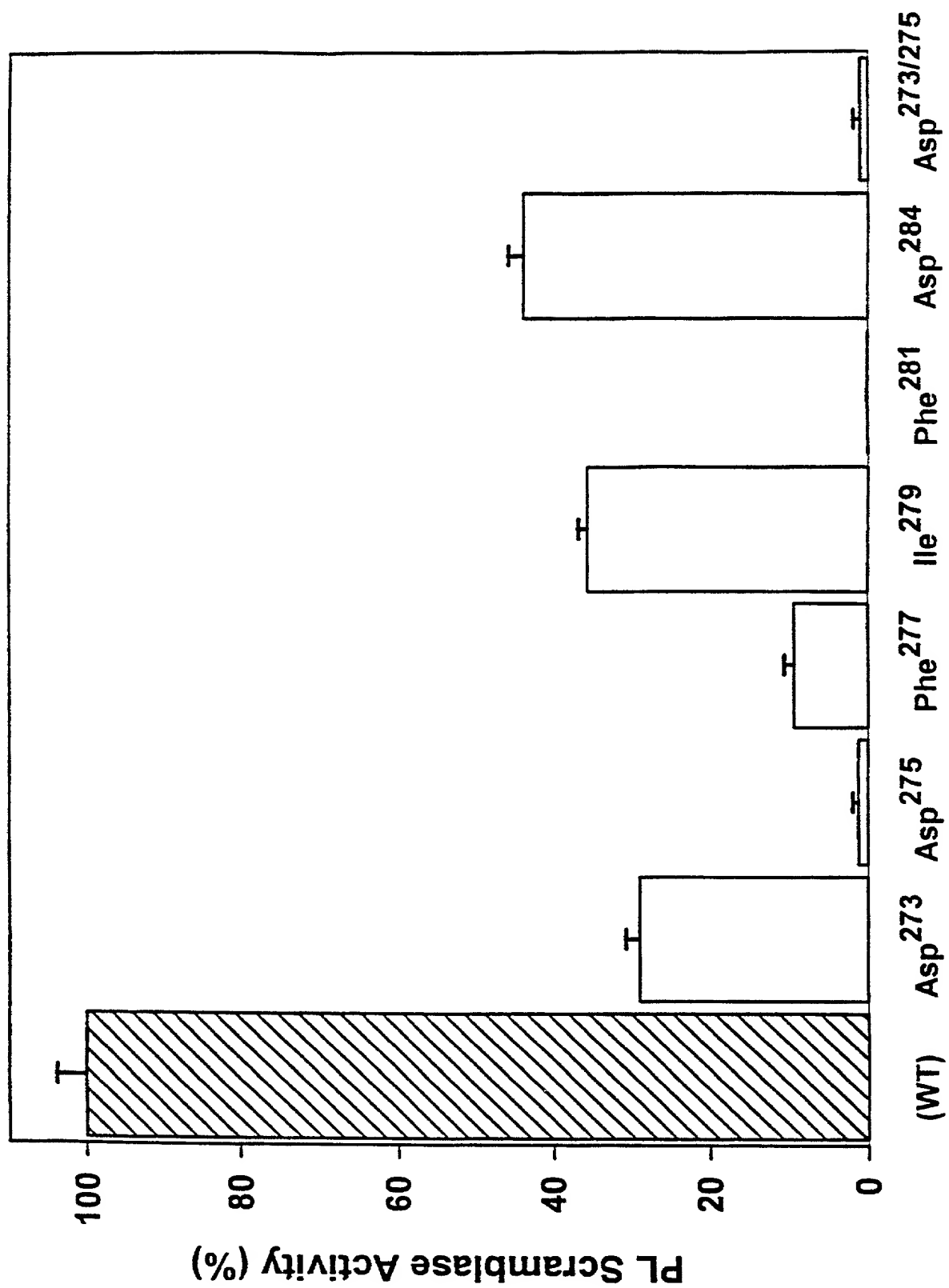


FIG. 6

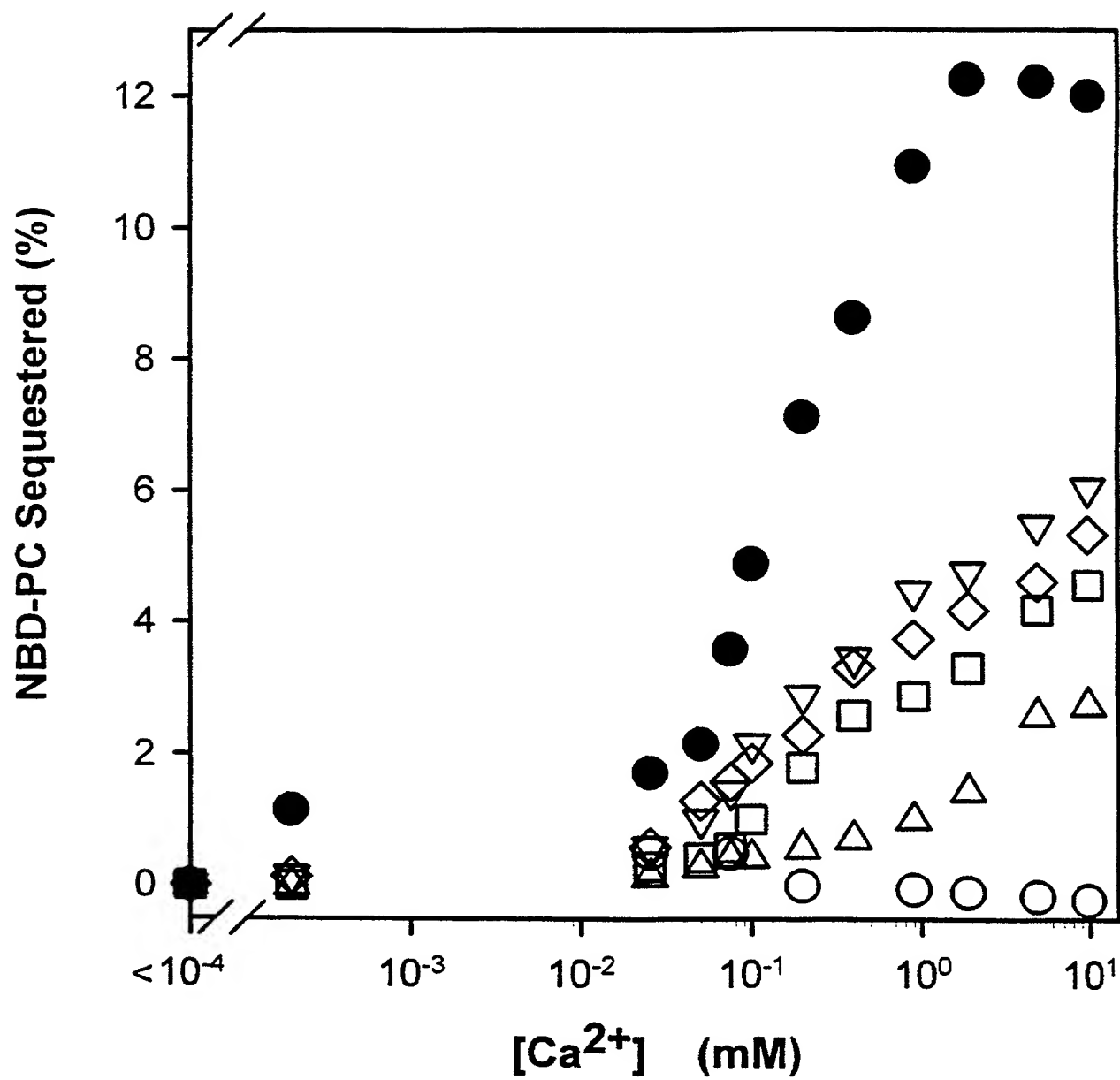


FIG. 7

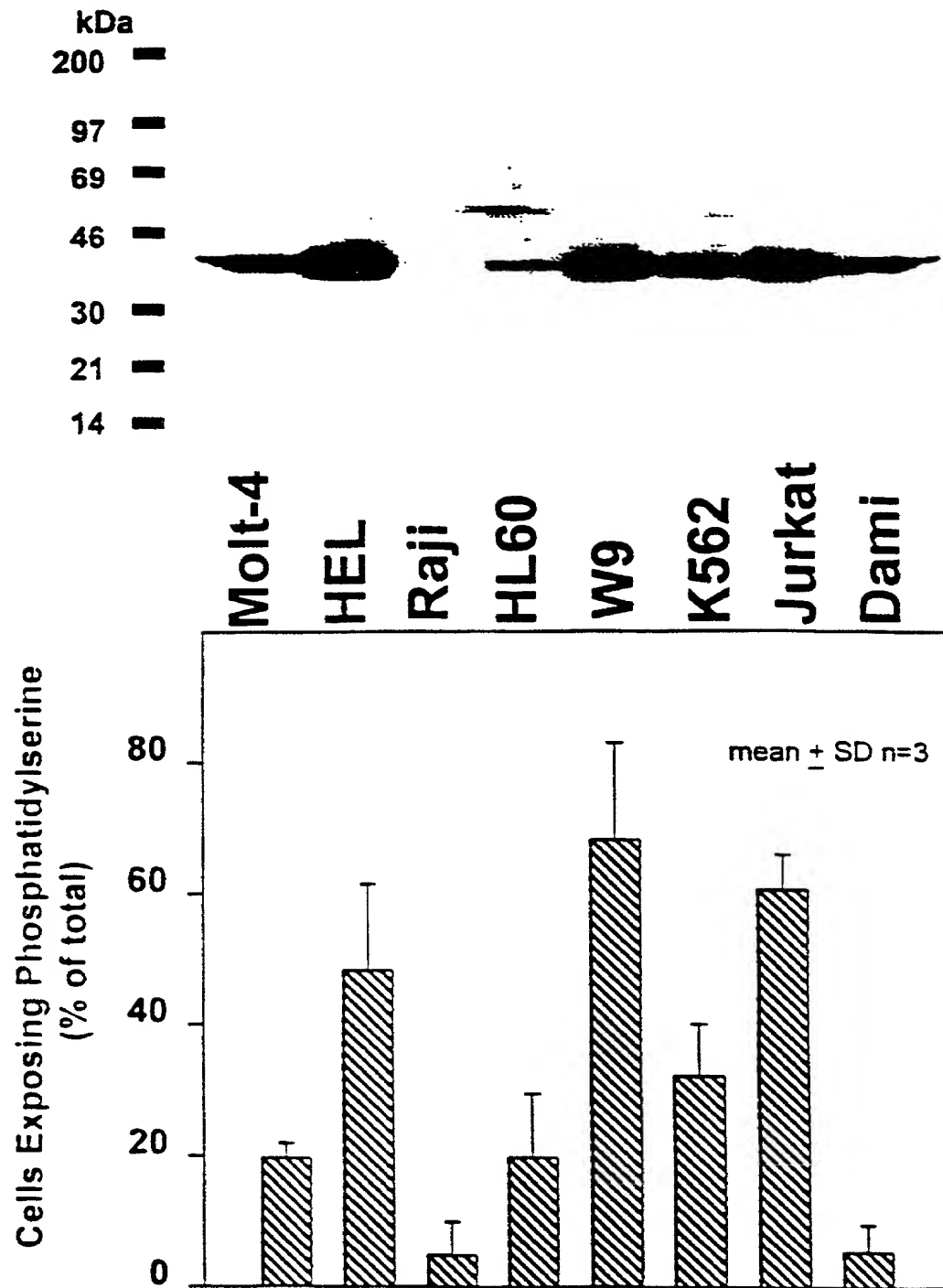


FIG. 8

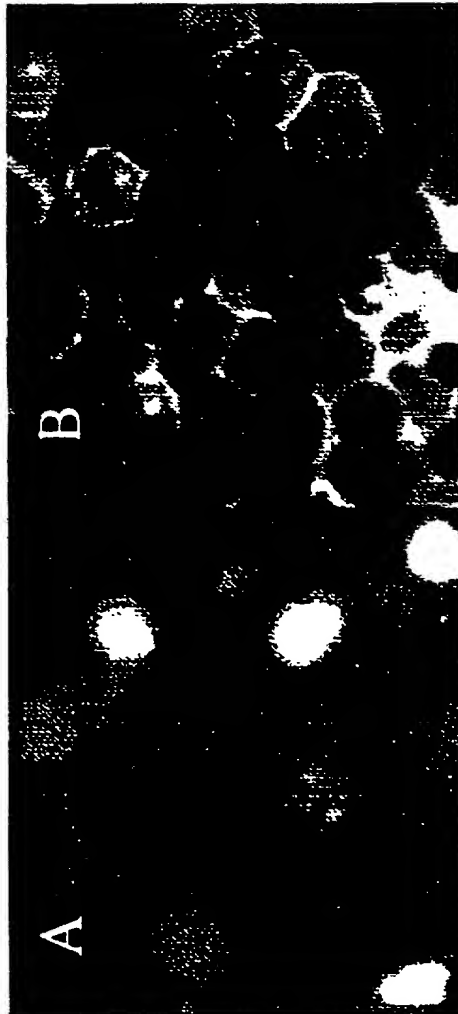


FIG. 9

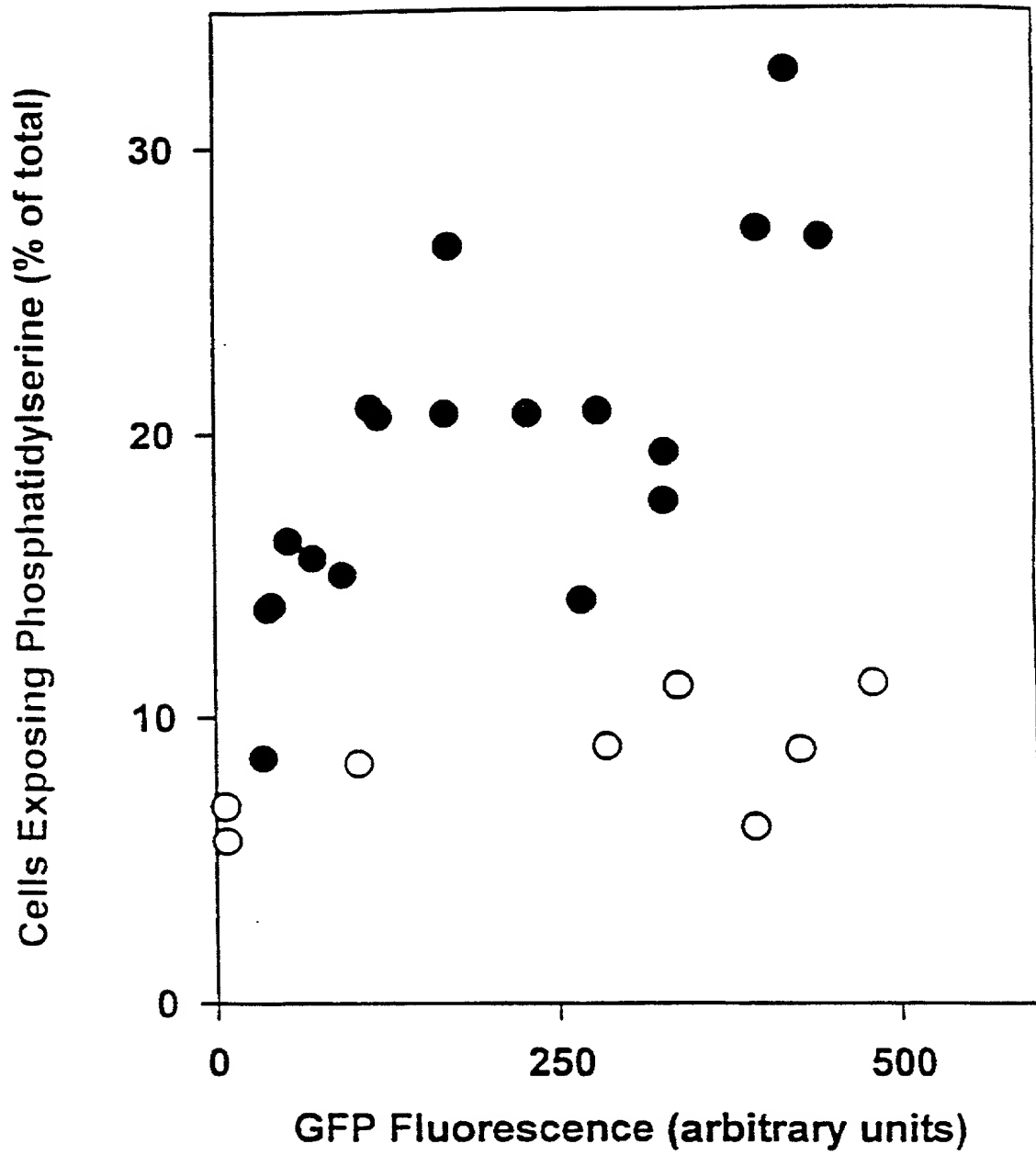


FIG. 10

Inactivation of PL Scramblase by Thioester Cleavage

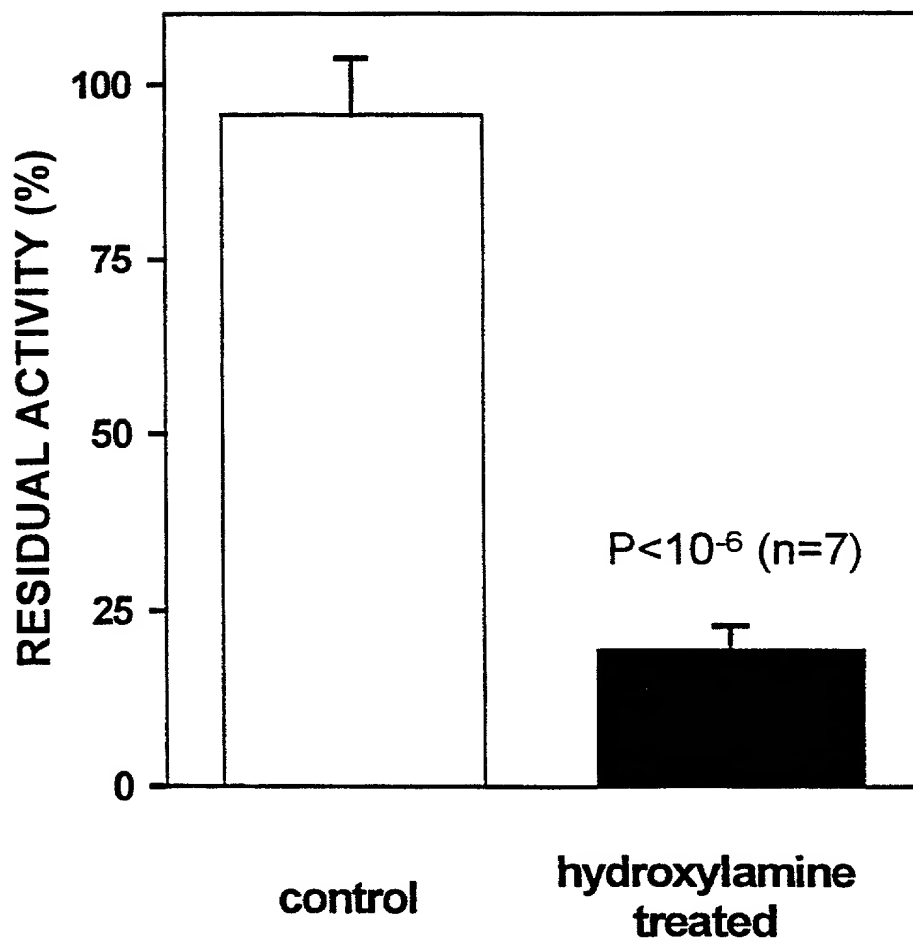


FIG. 11

**Metabolic Labeling of PL Scramblase with [^3H]-Palmitate
Reveals Covalent Thioester-Linked Fatty Acid**

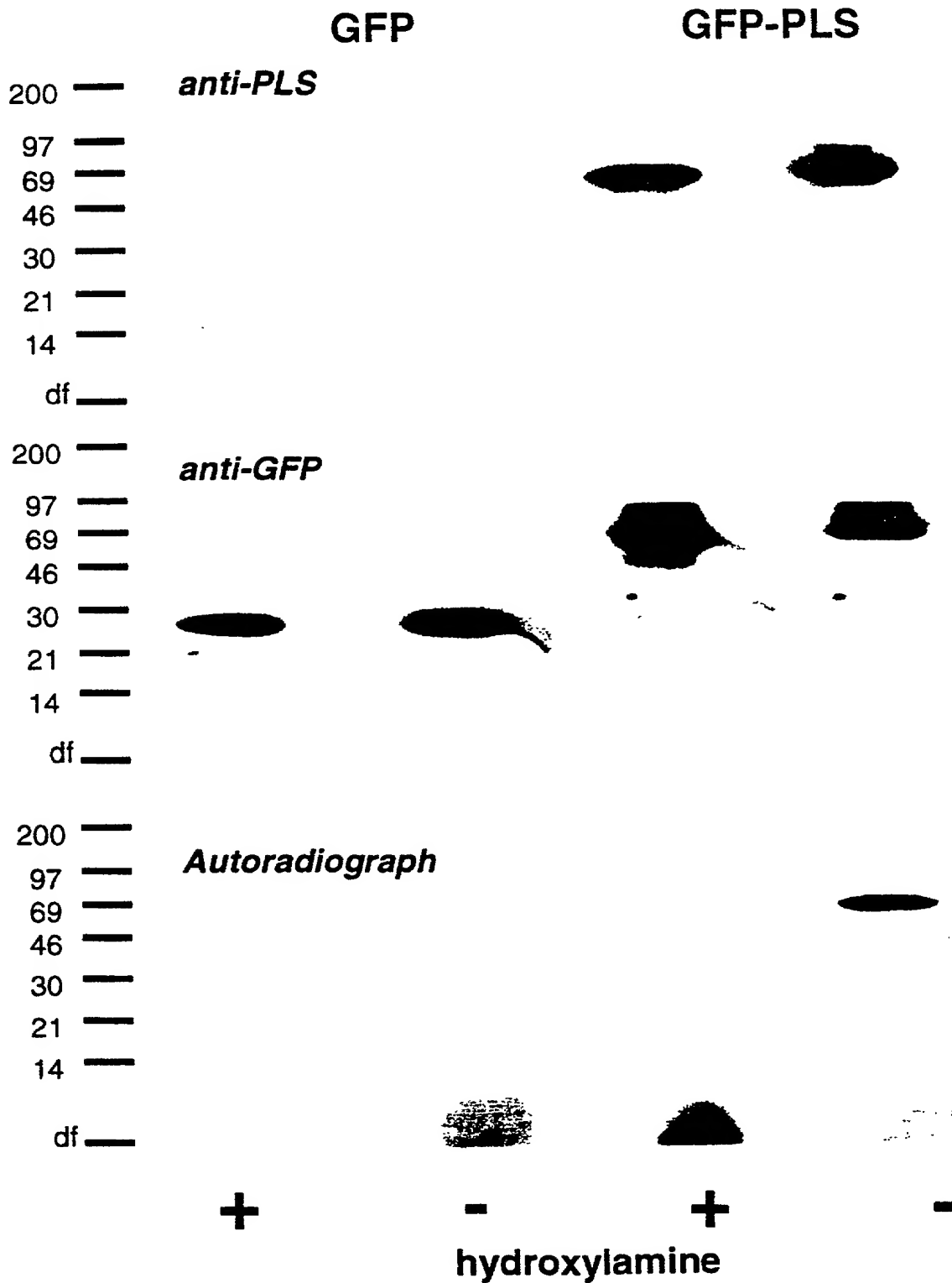


FIG. 12

TLC Analysis of [^3H]-Fatty Acid From Hydroxylamine-Treated PL Scramblase

SF —



OR —

palmitate

+

-

palmitate

FIG. 13

15 / 16
Cells Exposing Phosphatidylserine
(% of total)

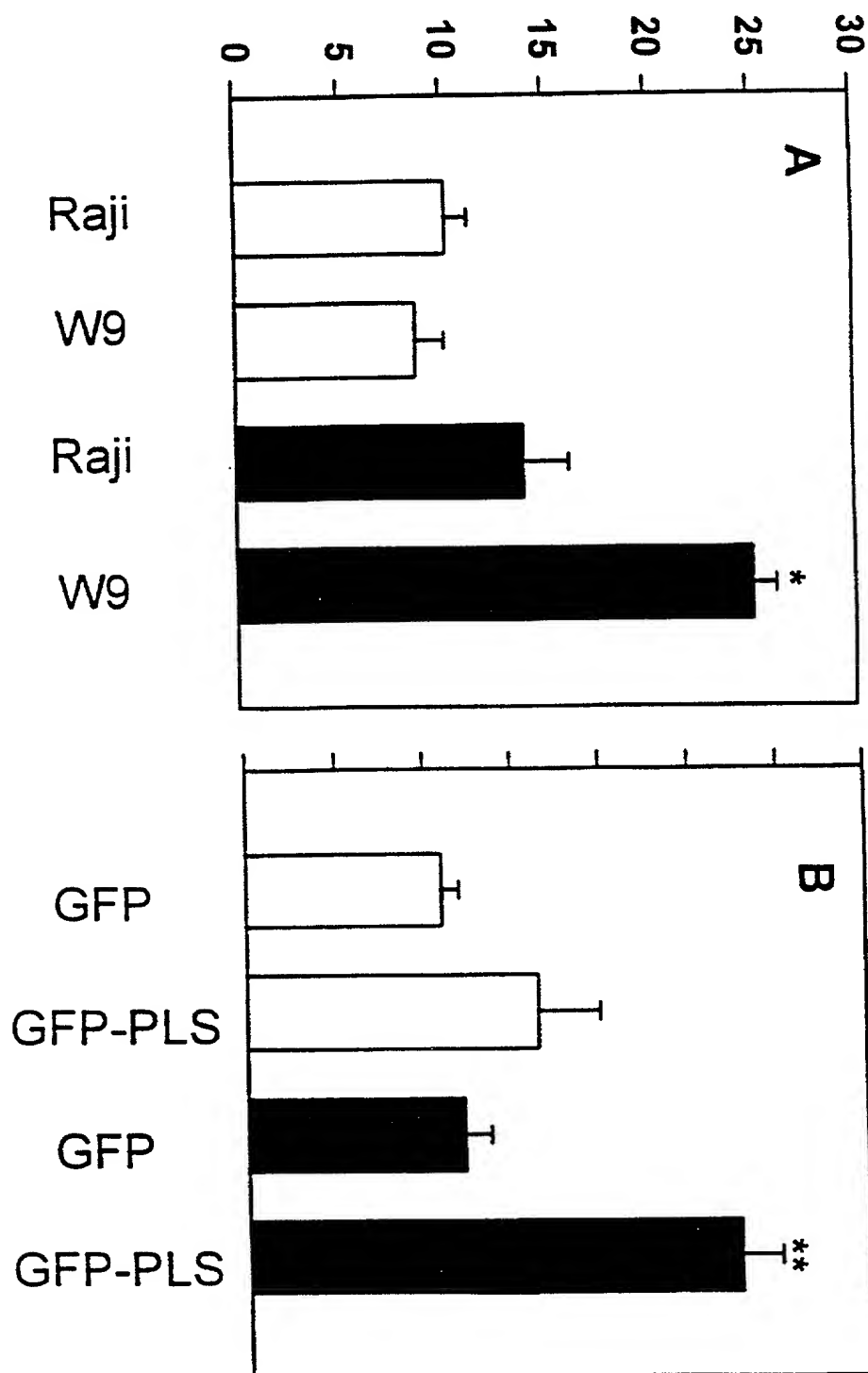


FIG. 14

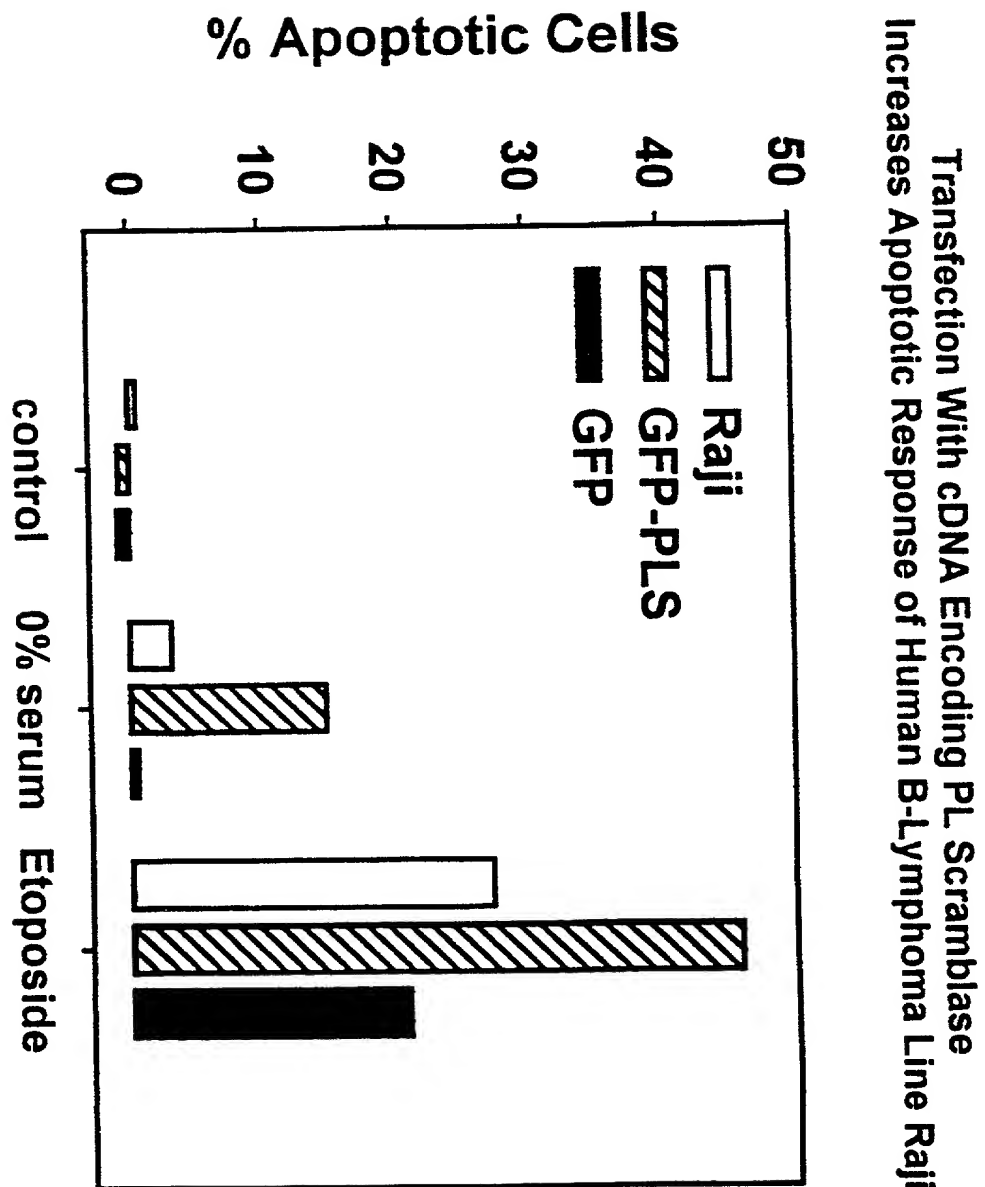


FIG. 15

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PTO/SB/01 (6-95)
Approved for use through 9/30/98. OMB 0651-0032
Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION <input type="checkbox"/> Declaration Submitted with Initial Filing OR <input checked="" type="checkbox"/> Declaration Submitted after Initial Filing	Attorney Docket Number	160180.90121
	First Named Inventor	Peter J. Sims
	COMPLETE IF KNOWN	
	Application Number	
	Filing Date	July 18, 2000
	Group Art Unit	
	Examiner Name	

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

METHODS AND COMPOSITIONS TO ALTER TISSUE SUSCEPTIBILITY TO IMMUNE INJURY, TO PROGRAMMED CELL DEATH, AND TO CLEARANCE BY THE RETICULOENDOTHELIAL SYSTEM

(Title of the Invention)

the specification of which

☐

is attached hereto

OR

☒

was filed on (MM/DD/YYYY)

07/18/2000

as United States Application Number or PCT International

Application Number

09/600,535

and was amended on (MM/DD/YYYY)

(if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations § 1.56

I hereby claim foreign priority benefits under Title 35, United States Code § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate or § 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not Claimed	Certified Copy Attached? YES	NO
PCT/US99/01087	PCT	01/19/1999	<input type="checkbox"/>	<input type="checkbox"/>	<input checked="" type="checkbox"/>
			<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
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			<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

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Additional foreign applications numbers are listed on a supplemental priority sheet attached hereto:

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Application Number(s)	Filing Date (MM/DD/YYYY)	<input type="checkbox"/> Additional provisional application numbers are listed on a supplemental priority sheet attached hereto.
60/071,950	01/20/1998	

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DECLARATION

Page 2

I hereby claim benefit under Title 35, United States Code §120 of any United States application(s), or §365(C) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application or PCT international application in the manner provided in the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. Parent Application Number	PCT Parent Number	Parent Filing Date (MM/DD/YYYY)	Parent Patent Number (if applicable)

☐ Additional U.S. or PCT international application numbers are listed on a supplemental priority sheet attached hereto

As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and all continuation and divisional applications based thereon, and to transact all business in the Patent and Trademark Office connected therewith

☐ Firm Name Customer Number or label

☒ List attorney(s) and/or agent(s) name and registration number below

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Barry E. Sammons	<u>25,608</u>	Richard T. Roche	<u>38,599</u>
J. Rodman Steele	<u>25,931</u>	Mark D. Passler	<u>40,764</u>
Nicholas J. Seay	<u>27,386</u>	Stanley A. Kim	<u>42,730</u>
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Carl R. Schwartz	<u>29,437</u>	Daniel G. Radler	<u>43,028</u>
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Keith M. Baxter	<u>31,233</u>	Steven J. Wietrzny	<u>44,402</u>
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Joseph W. Bain	<u>34,290</u>	David M. Kettner	<u>45,589</u>
Robert J. Sacco	<u>35,667</u>	Adam J. Forman	<u>P46,707</u>
Jean C. Baker	<u>35,433</u>		
David G. Ryser	<u>36,407</u>		

☐ Additional attorney(s) and/or agents named on a supplemental priority sheet attached hereto

Please direct all correspondence to ☐ Customer Number or label OR ☒ Fill in correspondence

Name Jean C. Baker

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Country USA Telephone 414) 277-5000 Fax (414) 271-3552

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Name of Sole or First Inventor: ☐ A petition has been filed for this unsigned inventor

Given Name Peter Middle Initial J. Family Name Sims Suffix e.g. Jr.

Inventor's Signature Peter J. Sims Date 8-28-00

Residence: Del Mar CA State CA Country US Citizenship US

Post Office 13386 Caminito Mar Villa

Post Office

City Del Mar State CA Zip 92014 Country US Applicant Authority

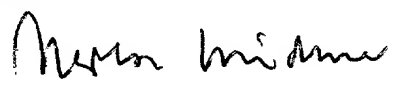
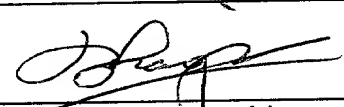
☒ Additional inventors are being named on supplemental sheet(s) attached hereto

008160-92500960

28-

1-00

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DECLARATION										ADDITIONAL INVENTOR(S) Supplemental Sheet			
Name of Additional Joint Inventor, if any:										A petition has been filed for this unsigned inventor			
Given Name	Therese				Middle Initial		Family Name	Wiedmer			Suffix e.g. Jr.		
Inventor's Signature										Date	8/28/00		
Residence:	Del Mar CA.				State	CA	Country	US			Citizenship	US	
Post Office	13386 Caminito Mar Villa												
Post Office													
City	Del Mar				State	CA	Zip	92014		Country	US		
										Applicant Authority			
Name of Additional Joint Inventor, if any:										A petition has been filed for this unsigned inventor			
Given Name	Ji				Middle Initial		Family Name	Zhao			Suffix e.g. Jr.		
Inventor's Signature										Date	8/28/00		
Residence:	Solana Beach				State	CA	Country	US			Citizenship	CN	
Post Office	309 North Solana Hills Drive, Apt. 105												
Post Office													
City	Solana Beach				State	CA	Zip	92075		Country	US		
										Applicant Authority			
Name of Additional Joint Inventor, if any:										A petition has been filed for this unsigned inventor			
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Inventor's Signature										Date			
Residence:					State		Country				Citizenship		
Post Office													
Post Office													
City					State		Zip			Country			
										Applicant Authority			
Name of Additional Joint Inventor, if any:										A petition has been filed for this unsigned inventor			
Given Name					Middle Initial		Family Name				Suffix e.g. Jr.		
Inventor's Signature										Date			
Residence:					State		Country				Citizenship		
Post Office													
Post Office													
City					State		Zip			Country			
										Applicant Authority			
Additional inventors are being named on supplemental sheet(s) attached hereto													

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